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56

CAPRYLIC ACID

A Monograph

Prepared for

Department of Health, Education and Welfare

Public Health Service

Food and Drug Administration

Rockville, Maryland

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FIRL Project No. 80G-C3378-01

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SUMMARY

Introduction

Caprylic acid is not a normal constituent of the mammalian free fatty acid (FFA) pool. Although the agent has been implicated as a Krebs cycle inhibitor, the effect is probably due to the accumulation of caprylic acid metabolites, which are cycle derivatives, with subsequent hormonally-regulated suppression of precursors.

Tissue Deposition

Rats fed a stock diet with 30% caloric energy from fats with C_8 - C_{10} acids for 29 days before and after liver by-pass were assayed for carcass fat. No depot evidence of caprylic was found before dietary treatment but 0.5% was detected before by-pass. One month post-anastomosis the carcass caprylic acid level had risen to 5.4% (167). Medium chain triglyceride (64% caprylic and 21% decanoic acids) given p.o. to rats and comprising 40% of the diet for 47 weeks led to insignificant changes in weight gain, mortality, liver and gut histology and liver, kidney, spleen, heart, adrenal, femur or testis weight. Epididymal fat and carcass composition contained less depot fatty acid than that expected of conventional fat diets. Only 0.4% caprylic was recovered in fat pad, while high levels of palmitic and oleic acids were detected, although the ration had contained only traces of these. Total liver lipid, triglyceride and cholesterol levels were lower than those for conventional fat diets, while liver phospholipid was higher (S-1). Calculated from dietary intake (uncorrected for endogenous loss) and fecal elimination (as percent of dietary intake), net medium chain triglyceride absorption for 35 weeks was 97 - 98%, which would be somewhat higher than that for conventional fats. *In vivo* confirmation of negligible caprylic label incorporation in portal vein, kidney, diaphragm, heart, brain, epididymal and perirenal fat was also reported (131). Fecal assay of a 5% caprylic acid dietary supplement found 3-5x less total lipid and up to 2 and 10x lower neutral ether and soap extract, resp., than in basic ration. The amount of fecal free caprylic acid or soap in the loose stools was therefore negligible (163).

Portal and Lymphatic Turnover

A single intragastric administration of labeled caprylic acid in rats led to a 1.5% thoracic duct and a 0.6% small gut capture after 8 hours. Since a combined fecal-intestinal fraction (mixed at sacrifice) was 5% of label, a 93% portal absorption from gut wall was inferred. The 8-hour ductal lipids consisted of 70% free acid and 29% triglyceride, while portal lipids included over 98% free acid with traces of triglyceride, cholesterol and phospholipid (69). It would follow that C_8-acid is not esterified by intestinal or liver enzymes but is catabolized to acetoacetate or CO_2, or is excreted in urine.

A mixed triglyceride (46% caprylic acid) was given p.o. in a case of pediatric chylothorax (41). Although patently absorbable, no serological evidence of caprylate was found in the systemic circulation even 3 1/2 hours post-administration of a 28 g daily dose and only 4% was recovered in chyle. Since nearly every triglyceride molecule included at least one caprylate group and the triglyceride lymphatic absorption factor (mg chyle acid/mg dietary acid) was only 0.7, nearly complete triglyceride hydrolysis would be anticipated. It would follow that caprylic acid was converted in gut or liver and did not pass the liver. This result is compatible with current theory of lipid turnover that primarily unsaturated longer-chain fatty acids are absorbed almost unmetabolized, while the shorter chain molecules are oxidized.

Tracer studies tend to support this. A single intraportal injection of carboxyl-labeled caprylic acid led to a 3-5% incorporation into liver lipid within 10 min and a 12% level within 15 min (130), a rate suggesting linear increases with time, which would require repeated liver passage. Assay of the lipid-soluble component resulted in a C_8:C_{16} value of 14/307, where the negligible C_8 compartment included 48% triglyceride, 38% FFA and 10% monoglyceride and phospholipid. Of the unesterified fraction, 18% was composed of C_6-C_{15}; 55%, C_{16}; 5%, C_{18} and 23%, C_{18-20} acids. Since over 80% of the label was in the carboxyl groups, the caprylic acid must have been oxidized to acetate or acetyl-CoA before incorporation into longer chain acids and subsequent triglyceride assembly. This outcome

contrasts with rat jejunum incubation data that only 9% caprylic acid was elongated in gut mucosa, while 91% of the uncatabolized fraction was recoverable intact and unesterified from the lipid-soluble fraction, relative to 96% C_{16} acid from palmitic acid ingestion (55). Lipid soluble fractions from rat liver slice incubation with labeled acid found 93% fatty acid, of which 83% was palmitic acid length or higher and less than 2% the original caprylic acid (131).

Hepatic and Hormonal Response

Fat droplet content in hepatocyte cytoplasm and nuclei and Golgi body hypertrophy were 6X that of controls in rats fed a 20% lipid diet for several months. In ATP and cytochrome C incubation with hepatocyte mitochondria, caprylate oxidation to acetoacetate was almost complete, with negligible CO_2 formation. The rate of acetoacetate formation per g mitochondrial nitrogen was also higher than control liver tissue (83). Incubation of normal liver and hepatoma-C954-host liver from mice with labeled acid led to a 50% label recovery in CO_2 respired, with 30% in the non-volatile fraction. In percent counts per min, 60 and 73% normal and tumor host liver non-volatile fractions, resp., were found in the β-hydroxybutyrate compartment, which included only 32% from the hepatoma. The glutamate and di- or tricarboxylic acid recovery for hepatoma-host liver was 40 and 13%, while for normal liver, 14 and 6%, resp.

Viscera and mesentery drained by the portal vein are not ketone body sources, but mesenteric fat contributes ketone body precursors as FFA to liver. Splanchnic ketogenesis is probably a measure of both mesenteric fatty acid-release and true liver ketogenesis. Approximately 9% of caprylic acid was converted to ketone bodies and net splanchnic ketogenesis was 33% lower than controls after i.v. infusion of 1.5% acid with glucose in humans. Ketogenesis from the same concentration of acid in combined glucose-insulin solutions was 11%, or 25-30% below control values; arterial ketone levels were also lower (86). These values contrast markedly with those in acute transient insulin hypoglycemia and exogenous caprylate in which 3-hour posthypoglycemic splanchnic ketogenesis reached 65% above control levels. The difference would suggest either increased liver caprylate catabolism or accelerated release from adipose tissue and delivery to liver.

If carbohydrate sparing of intermediate chain oxidation is assumed as minimal, the net glucose effect would be an interruption of intracellular C_8-chain oxidation and not a reduction in tissue utilization of ketone bodies. However, a glucose challenge p.o. 3 days after i.v. caprylate failed to inhibit C_8 oxidation, while an i.v. C_8 challenge to a prior i.v. C_8 dose had no effect on the amount or rate of C_8 oxidation (134). Confirmation was found in caprylate-glucose duodenal infusion data in humans without diabetic history (84). The C_8-chain acid significantly potentiated glucose-induced increases in serum insulin for both healthy and cirrhotic subjects. Serum caprylate was detectable within 10 min after infusion in both subjects types, peaking at 30 min; normalization was slower in the cirrhotic group. However, serum FFA was not elevated nor was liver glucogenesis affected. It is not known whether these effects are attributable to a direct pancreatic effect or an indirect outcome following release of an unknown insulin-inducing factor during absorption in the gut wall (89).

Ketogenesis and Alternate Pathways

Dogs receiving 2 high i.v. doses of isotonic caprylate at roughly 5 day intervals before and during initial fasting showed insignificant ketogenesis and hypoglycemia. A third caprylate challenge 5 days before the hunger period ended found a transient massive increase in ketonemia and slight hypoglycemia. A fourth challenge at the end of fasting resembled the pre-fast response except for a more exacerbated hypoglycemia (91). Subsequent doses during consecutive fattening and high-protein diets triggered only moderate transient ketonemia with increasingly prolonged normalization; the hypoglycemia was equally severe for each case and was slow to normalize. Each caprylate administration at any time and for all nutritional states elicited an immediate and highly transient initial hyperglycemia which reflected glucose mobilization via glycogenolysis. The ketonemia was simultaneous with the hypoglycemia and was a measure of the amount of subsequent glycolysis, whose rate must be sufficiently rapid to explain the ephemeral hyperglycemic interval (91). The degree of gluconeogenesis, if any, remains unknown.

Other rat liver incubation studies found 20% of the caprylate administered responsible for ketone body formation and 4-5% oxidized to CO_2, compatible with β-oxidation and subsequent condensation of 2-carbon segments (161). Liver assay after intraportal labeled acid infusion found a C_8/C_{16}

ratio of 214/15 for CO_2 generation (130). However, liver ablation did not significantly reduce the amount of labeled CO_2 in expired air, suggesting preferentially competitive extrahepatic oxidative pathways. CO_2 recovery - amounting to 15-20% of label - was also indifferent to portal of acid entry. Label recovery from fasted rat liver slice incubation was 2x lower for fasting than for fed animals (131), implying the constraint of nutritional status. Incubation of 2-3 μM of acid with hemidiaphragms from rats fed either carbohydrate or fat diets found a 14% increase in oxygen consumption for both rations. A kinetically equivalent result was obtained at 3-5 μM/ml acid concentrations with stock diets, while 3-5 μM of acid inhibited oxygen consumption toward the end of a 2-hour incubation during which C_8 uptake was maximal (60). In general, oxygen consumption of rat liver slices during caprylic incubation was significantly lower than calculations of complete oxidation would predict (161). The presence of unknown non-oxidative pathways for caprylate catabolism may be inferred. Caprylate disappearance, ketogenesis and oxygen consumption as measured by caprylic oxidase activity in rat liver homogenates after prolonged steatogenous rationing (without choline or vitamin B_{12}) was 30% below that for standard feeds (144). Since the ketogenesis/caprylate disappearance quotient was ca. 0.8 for both diets, additional support for alternate pathways is indicated.

Medium chain triglyceride, containing 80% caprylic and 17% decanoic acids, led to highly significant elevations in both serum ketone and insulin and a moderate fall in serum glucose in human dietary studies at 1000 mg/kg levels (112). In particular, the ketone level - which rose 9x over controls - remained 8x higher for at least 5 hr, while the hypoglycemia and hyperinsulinemia were 50% normalized over that interval. There was no significant serum caprylate elevation. Since predominantly caprylic acid medium chain triglycerides elicited a greater serum hyperinsulinemia than long chain triglycerides, the effect cannot be attributed to release of a fat-mediated gastrointestinal hormone. However, it is not known whether the hyperinsulinemia follows from the hyperketonemia due to portal catabolism or the escape of some caprylate fraction from hepatic catabolism. The latter case would tend to confirm the presence of alternate extrahepatic pathways.

Protein Synthesis and Membrane Perturbation

Rat incubation studies with intact, hemidissected or hemidiaphragm and labeled leucine with or without insulin and glucose showed that amino acid uptake from medium was higher, but label incorporation into protein lower, in the presence of 0.5, 1 or 3mM caprylic acid (24). It would follow that alteration of amino acid metabolism occurred after membrane transport. Inhibition of protein synthesis, even at low concentrations, would suggest easier membrane penetration by the caprylate component (relative to higher chain acids). By inhibiting protein synthesis, or its rate, the resulting high intracellular accumulation would be expected to increase the amino acid pool available for gluconeogenesis during stress or carbohydrate deficit. However, caprylic acid at high concentrations (3mM) was found to be membrane-perturbing, based on the behavior of a non-utilizing amino acid as an index of cell penetration. In bovine rbc studies molecular size was the limiting factor for penetration of C_6 - C_8 acids, with lipid solubility determinant for C_1 - C_3; C_4 - C_5 represented a compensating effect between the 2 factors. The rate and amount of caprylate penetration was greater than C_1 - C_3, but less than C_5 acids (53). There was no difference in protein absorption or age-dependent fall in calcium ion retention between conventional fat and predominantly caprylate medium chain triglyceride diets (S-1). The acid was also found to potentiate tryptic digestion of ovalbumin to the same degree as heat denaturation, while 5x higher concentrations inhibited tryptic digestion, probably due to denaturation of both substrate and enzyme (77). Ovalbumin hydrolysis was reduced 20% by adsorption of 3 mg caprylate on pepsin micelles (155). Caprylic acid was exponentially bactericidal against enteropathogenic *E. coli* at 0.3-0.5% (93).

Lipemogenesis

The relation between lipemogenesis, thrombogenicity and atherogenicity is paradoxical.

A high fat diet including 5% caprylic acid fed to dogs elicited a degree of endotoxin-induced thrombosis equivalent to that of C_{12}, but greater than C_{14} and lower than C_{16} or C_{18}, acids. The thromboses exhibited after 6 weeks of high fat ration were histologically equivalent to those normally occurring

in human coronary systems only after age 40. However, plasma clotting time was normal. Serum cholesterol and triglyceride were equivalent to C_{14}, less than C_{16} and higher than C_{12} or C_{18} acids (119). In contrast, total plasma cholesterol was lower in human males, but not females, than control subjects after 35 weeks on a triglyceride diet (75% C_8 and 25% C_{10}) (S-1). That caprylic acid is not hyperlipemic according to the above data is at least compatible with current theory which predicts that saturated acids below C_{12} do not increase serum cholesterol. There would also seem to be no discriminable relation between lipemogenesis and thrombogenicity.

Reproductive effects

Rats of both sexes 12 weeks of age received dietary medium-chain triglyceride (75% caprylic acid, 25% decanoic acid) 3 weeks before mating. There was no effect in F_1 litter size or birth weight between the triglyceride and low fat or conventional diet. As with F_0, F_1 was fed conventionally for 12 weeks until switched to triglyceride, high fat or low fat feeds. The composition of F_0 milk included only 6.5% caprylic and 17% decanoic (with 75% C_{12} or higher), confirming that the triglyceride rats had to synthesize 75% of milk fat fatty acids. Milk secretion in F_1 was significantly lower. There was also a higher mortality (20-22%) during lactation for F_2 on triglycerides (S-1).

Toxicity

Cheyne-Stokes respiration and general disorientation followed an initial massive i.v. infusion of ca. 3750 mg/kg caprylate in dogs, with adaptation following subsequent challenges (S-1). A 5% concentration in the diet of dogs led to a modest catharsis whose semifluid stool formation was attributed to the mixture of small caprylic acid doses with large amounts of non-titratable material (163). Flushes, nausea and joint and back pain were reported for at least one human receiving a single 28.5 mg/kg treatment i.v. (134) - a non-specific response not incompatible with allergic or anaphylactic reactivity.

Conclusion

The gastrointestinal fate of caprylic acid is unexceptional. Hydrolysis of caprylate-containing triglycerides in the gut mucosa proceeds via intraluminal bile emulsification, bile complexation with the liberated fatty acids and their

active transport into the villi with subsequent lacteal or portal uptake. Selective interception of the C_8 molecule by portal venules probably follows from the relatively short chain length and relatively faster venous drainage. However, there is accumulation of a free caprylate pool in the intestinal mucosa due to the low activity of esterifying enzymes *in situ*, with negligible chain elongation. Pool depletion would require that portal transport vary linearly with time, with complete depletion requiring repeated liver passage. There is no evidence for a caprylic compartment in tissue depot fat, even after liver by-pass, nor significant fecal elimination. Serum caprylate levels were vicariously negligible.

It follows that caprylic acid undergoes (at least) extensive portal burn. Tracer data confirms facile oxidation prior to chain elongation and triglyceride assembly. The maximum rate of oxidation is not established in humans but the ketogenic outcomes suggest almost instantaneous acetate or acetyl-CoA formation compatible with known intracellular citrate accumulation. The relative degrees of ketonemia and CO_2 accumulation fluctuate, probably consequent to feedback interception by other available fatty acid compartments. However, there is evidence from incubation systems that oxygen requirements for caprylic oxidation are considerably higher than actual oxygen consumption, indicating the presence of alternate non-oxidative pathways. There is also evidence of kinetically equivalent extra hepatic oxidative pathways, which the hormonal data would support.

Moreover, consideration of data confirming dubious or idiopathic manifestations of toxicity leads to the conclusion that intake levels even several orders of magnitude higher than those postulated by NAS/NRC consumer exposure information would not lead to tissue insult. There is reason to believe that caprylic acid serves as a human growth support, sparing carbohydrate via facile caloric availability and fat by freeing longer chain acids for storage.

CHEMICAL INFORMATION

I. Nomenclature

A. Common names: caprylic acid; n-caprylic acid; octanoic acid; octic acid; n-octoic acid; octylic acid; n-octylic acid

B. Chemical names: octanoic acid; 1-heptane carboxylic acid

C. Trade name: Neo-Fat 8 (origin unknown)

C. CAS Reg. No.: 124072

II. Empirical Formula

$C_8H_{16}O_2$

III. Structural Formula

$CH_3(CH_2)_6CO_2H$

IV. Molecular Weight

144.21

V. Specifications

A. Food Chemicals Codex

Acid value: 366-396

Saponification value: 366-398

Titer (solidification point): 8°-15°C

Limits of impurities:

arsenic (as As) - 3 ppm

heavy metals (as Pb) - 10 ppm

iodine value - 2.0

ignition residue - 1000 ppm

unsaponifiable matter - 2000 ppm

water - 4000 ppm

B. Chemical

Mallinckrodt, Fisher, Baker and MC & B all report a m.p. of 15-16°C for their high purity grade; no other specs are given.

VI. Description

A. General (from Food Chemicals Codex): a colorless, oily liquid having a slight, unpleasant odor and a burning, rancid taste.

B. Physical properties:

m.p. = 16.3°C

b.p. = 239.7°/760 mm; 110-1°/4 mm

d_4^{20} = 0.910; d_4^{80} = 0.8615

n_D^{20} = 1.4278

solubility-

water - 0.068g/100g at 20°

ethanol - miscible

chloroform - miscible

acetonitrile - miscible

ether, carbon disulfide, acetic acid, petroleum ether - freely soluble

C. Stability: stable under normal conditions

VII. Analytical Methods

A. Gas chromatography

1. Ref. 5

Solvent extraction was used to separate the medium chain FFA from raw soybeans. They were separated on a column of Silicone DC-550/Stearic acid (95:5) and detected by thermal conductivity Retention time on a 3 x 1400 cm column at 140°C was 14 min.

2. Ref. 66

A model system (mixture FFA) was separated on a glass column packed with diethylene glycol adipate (DEGA) on firebrick. Detection was via combustion over CuO and thermal conductivity detection of the CO_2 formed. The method detected 0.2 mg of caprylic acid and would probably work with less. Large proportions of water in the sample do not interfere.

3. Ref. 67

A sample of FFA from a milk fat saponification mixture was separated on a glass column (5' x 4 mm) packed with 20% "Carbowax 20M terephthalic acid" on silanized Chromosorb W. The C_4 - C_{12} acids are analyzed in about 10 minutes at a column t = 235°C with argon carrier gas; detection was via flame ionization.

4. Ref. 147

A sample of beer or wort, saturated with salt, pH 2.0, was extracted with hexane-butanol (99:1), placed on a silicic acid-KOH column, washed with ether, eluted with phosphoric acid and esterified with n-butanol. The esters were separated on a diethylene glycol succinate (DEGS) column and detected by flame ionization.

5. Ref. 150

A model system (solution of FFA, C_1-C_8) was analyzed after conversion of the acids to their anilide derivatives. Separation was via a 12' x 3 mm column dodecylbenzene sulfonate on Chromosorb G and was followed by flame ionization detection.

6. Ref. S2

The FFA of butteroil and Provolone cheese were isolated by solvent extraction and column chromatographic cleanup. Conversion to the butyl esters was followed by separation on a 150 x 0.31 cm stainless steel column packed with 15% DEGS on silanized Chromosorb W. Detection was via flame ionization. A concentration of 20 ppm was determined accurately.

B. Paper chromatography

1. Ref. 51

FFA in tea oils were chromatographed with a 1:1 mixt. of butanol and 1.5 N ammonia. The indicator spray was chlorophenol red and gave yellow spots on a violet background.

2. Ref. 157

A model mixture of FFA was separated on polycaprolactam-treated treated paper with cyclohexane as solvent. Detection was via bromphenol blue which produced yellow spots.

C. Thin-layer chromatography

1. Ref. 37

A model system (lipid extract) was separated on Silica Gel G, the solvent system being chloroform-methanol-water (60:30:1) for the polar fraction. The following spray sequence was used: (1) 2',7'-dichlorofluorescein; (2) alcoholic $AlCl_3$; (3) aqu. $FeCl_3$; it yielded a rose-violet spot on a pale yellow background. The lower limit of detection was 2μg.

D. Titrimetry

1. Ref. 85

A model system was used. Individual FFA were titrated potentiometrically with sodium methoxide in various non-aqu. solvents. The error varied from -0.32% (in β-ethoxy-ethanol) to +1.1% (in methanol-benzene).

VIII. Natural Occurrence

Source	Level (ppm)	Ref.
Vegetable		
raw soybeans	trace (not organoleptically significant)	(5)
Dairy products		
butteroil	15-20	(S2)
cheese		
Gouda (Wisconsin)	15-36	(71)
Feta (Italian and Greek)	5-422 (in a very rancid sample)	(38)
Provolone (6-month (extra piccante	194 1135	(S2)
Beverages		
beer	8	(122)
beer (ordinary, no-hops; all malt	7-15 10;10	(147)
brandy distillate	3.2-12.6	(56)
tea (essential oil Russian black)	present only in fermented leaves	(51)

BIOLOGICAL DATA

I. Acute toxicity

1. Ref. 134

species	sex/no.	route	dose	measurement
human	?/1	i.v.	29 mg/kg	ED

A 2 g i.v. infusion of caprylic acid (dissolved in aqueous bicarbonate) given to a human volunteer was accompanied by flushing, nausea, joint and back pain and resulted in a moderate depression of oxidation of a subsequent tracer dose of labeled caprylic acid. Doses of 1 g failed to elicit these reactions.

II. Short-term studies

1. Ref. 119

Male Holtzman rats (7) of 150 - 170 g body weight were fed 5% caprylic acid for 6 weeks in a high fat diet of butter (32%), casein, cellulose, cholesterol (5%), salt mixture, sodium cholate, sucrose and vitamin mixture. Serum cholesterol and triglyceride levels were ca. 720 and 156 mg/100 ml, resp., which were approximately those of myristic acid fed animals but less than those for palmitic acid and more than those for stearic and lauric. Plasma clotting time was normal. The degree of thrombosis (induced by *S. typhosa* lipopolysaccharide) was ca. equal to that with lauric acid animals, but more than with myristic and significantly less than that with either palmitic or stearic. Survival time after endotoxin treatment was ca. 9 hours, which contrasted with 13 for lauric, 7 1/2 for myristic, 11 1/2 for stearic and only 3 1/3 for palmitic acid fed animals.

2. Ref. S1 (triglyceride)

A medium chain triglyceride (MCT) containing 75% caprylic and 25% decanoic acid was given in the diet to 15 male and 15 female Wistar rats for 47 weeks. Weekly fecal assays were run before sacrifice at termination of feeding study. Weight gain for both sexes was slightly below that for other fatty diets given to different experimental groups. Mortality variations were negligible. Organ weights of liver, kidney, spleen, heart, adrenal, femur and testis were unremarkable. Histology of liver and intestine was normal. Epididymal fat pad (2% of body weight) and carcass composition (22%) exclusive of liver, heart, epididymis or gastrointestinal tract were less than fat storage values after conventional fat diets. Although the MCT involved comprised 85% of the diet, only 0.4% caprylic acid was recovered in fat pad; high levels of palmitic and oleic were recovered, however, although only traces may have been in the diet. Higher chain acids in diet were generally incorporated directly into depot fat, while C_8 sources were apparently catabolized to smaller units, thus escaping tissue deposition. Approximately 30% of the MCT molecule was resynthesized to C_{16} and 56% to C_{18} acids in epididymal fat pad. It was highly

probable, that a facile C_8 burn would free higher chain acids for depot functions under conditions of normal dietary intake.

Total plasma cholesterol was generally lower in male, but not female, animals on MCT than conventional fats for the first 35 weeks. Assay from intracardiac puncture at sacrifice after an 18-hour fast found 100 and 27 mg/100 ml plasma, resp., for total and free cholesterol, which values exceeded those after conventional fats. The esterified component of 73 mg/100 ml was also highest, except for coconut oil diets, but the percent esterification was lowest of all the fat diets used. Total liver lipid after MCT was also lowest of all the diets, as was liver cholesterol (2.5 mg/g); liver phospholipid was highest (26 mg/g). Based on calculations from fecal excretion and dietary intake, the net MCT absorption for 35 weeks was 97-98%, a value which exceeded those for conventional fats.

3. Ref. 75

Species: rat
Strain: albino
Sex: male
Age: weanling
Duration: 35, 42, 56 days
Route of administration: p.o. (diet)

An 8% sodium caprylate addition to diets with adequate essential fatty acids altered normal nitrogen excretion and energy patterns, although efficient caloric and protein utilization remained unaffected.

III. Long-term studies

No information available

IV. Special studies

1. Ref. S1 (triglyceride)

Young adult male and female McCollum-Wisconsin rats received dietary triglyceride (MCT) containing 75% caprylic and 25% decanoic acid for 3 weeks before mating. There was no difference in F_1 litter size or birth weight between MCT and a conventional or low fat diet; mortality during lactation was somewhat higher after MCT. F_1 rats were dieted as the F0 generation had been until 12 weeks of age when diets were switched such that the MCT subgroup subsequently received either MCT, high fat or low fat. Although litter size and birth weight were similar for each subgroup, mortality during lactation was high (20-22%) for those MCT groups receiving either subsequent MCT or low fat feeds. Further growth of the survivors was unremarkable.

There was no diet-associated differences in F_0 lactation, while F_1 milk secretion after MCT was significantly lower, evidently related to intake of MCT. Composition of F_0 milk included only 6.5% C_8 and 17% C_{10} acids. The percent of C_{12} acids was 10; C_{14}, 11.5; C_{16}, 30; C_{18}, 5; $C_{18:1}$, 12 and $C_{18:2}$, 6.5, confirming that MCT rats had to synthesize a large component of milk fat fatty acid.

2. Ref. 93

The cidal effect of various caprylic acid concentrations up to 1.8% on *E. coli* B, enteropathogenic *E. coli* OB E 0127:B8, *S. sonnei* 9453 and *S. sonnei* 6761 in a chemically defined medium (pH 7.0) was assayed by standard plate counting. Caprylic acid effectively sterilized all cultures at the following concentrations: *E. coli*, B 0.3%; enteropathogenic *E. coli*, 0.5%; *S. sonnei* 9453, 0.5% and *S. sonnei* 6761, 0.5%. The toxicity was exponential in each case. No mechanistic explanation is provided for the caprylic dose-response, but the much weaker, non-linear inhibition by acetic and propionic acids was not attributed to pH effects alone.

BIOCHEMICAL INFORMATION

I. Breakdown

No information available

II. Absorption-Distribution

1. Ref. 22

Liver and hepatoma C_{954} slices from C_{57}-Leadem mice were incubated with 5 μM 1-C^{14}-caprylate in calcium-free Krebs-Ringer phosphate buffer (pH 7.4) and the label recovery assayed. Expressed as percent of label recovered, results were as follows (Table I):

Tissue	Respired CO_2	Non-volatile fraction	Volatile fraction	Total
Normal liver	48.4	32.5	12.8	93.7
Hepatoma	38.7	22.5	2.4	63.6
Tumor-host liver	49.4	36.7	7.3	93.4

Accordingly, about 50% label incorporation occurred in normal and host liver and about 40% in tumor. Within the non-volatile fraction (reacted substrate) significant levels were as shown in Table II (see next page).

Traces of urea in both liver specimens and pyrrolidine-COOH in normal liver were also found. All of these compounds are known to be tricarboxylic acid cycle derivatives but not proper parts of the cycle itself.

In further substrate studies, the C_8 chain was preferentially selected over acetate, as follows in Table III (as % of incubated label, in bicarbonate buffer):

	Respired CO_2	Acetoacetate	Nonvolatile fraction	Volatile fraction
Host liver				
1-C^{14}-acetate	8.8	1.1	4.9	73
1-C^{14}caprylate (0.002M)	20.5	16.5	43	24
1-C^{14}-acetate + caprylate	3.1	0.67	3.3	77
Hepatoma				
1-C^{14}-acetate	3.3	0.15	1.4	79
1-C^{14}-caprylate (0.002M)	18.0	1.9	7.3	77
1-C^{14}-acetate + caprylate	1.4	0.1	0.7	80

TABLE II (values in cpm)

Moiety	Normal liver		Hepatoma C_{954}		Host liver	
	% non-vol.	% incubated	% non-vol.	% incubated	% non-vol.	% incubated
β-hydroxybutyrate	58.5	19	31.7	7.2	73.1	26.8
glutamate	15.1	4.6	40.3	9.1	2.6	1.0
glutamine	6.7	2.2	0.8	0.2	6.3	2.3
di- and tri carboxylic acids	6.0	2.0	12.5	2.8	4.1	1.5
aspartate	3.3	1.1	3.5	0.8	2.1	2.5
glucose	3.2	1.0	1.3	0.3	6.9	2.5
alanine	2.2	0.7	4.6	1.0	1.9	0.7
lactate	1.1	0.4	1.5	0.3	1.0	0.4
glutathione	0.8	0.3	3.5	0.8	----	----

Analysis of the major nonvolatile compounds found 58% β-hydroxybutyrate, 12% glutamine, 8% glucose, 7% glutamate and 6% di-and tricarboxylic acids in host liver after caprylate incubation. Glutamate (34%), di- and tricarboxylic acids (19%), β-hydroxybutyrate (12.5%), lactate (12%), aspartate (7%) and glutamine (5%) were the main compounds derived from hepatoma incubation with labeled caprylate. In general, there was a modest decrease in label recovery from caprylate, ostensibly due to its greater ketogenic potential, relative to recovery from acetate. Labeled acetate was found to contribute to the ketogenicity of C_8 during incubation with unlabeled caprylate, a property peculiar to even-chain fatty acids. Metabolic shifts from host liver characteristic of the hepatoma included the following: (a) reduced ketogenesis, (b) higher levels of non-volatile label, especially with glutamate and polycarboxylic acids and (c) low glucose label - responses which were more prominent in phosphate than bicarbonate buffer. The metabolic direction of hepatoma was unaffected by substrate concentration, nutritional status, tumor age or buffer type. Table III also confirmed that caprylate is a major hepatoma as well as normal, Krebs cycle inhibitor, probably due to suppression of acetate stimulation consequent to competitive activation of acyl S-CoA.

2. Ref. 69

Species: rat

Strain: Wistar

Sex: male

Age at start of experiment: adult

Duration: 8 hours

Vehicle: emulsion of 50 mg albumin, 288 mg Na taurocholate to 3 ml saline

Dose schedule: a single intragastric administration of 150 mg

Route of administration: intra-gastric

Samples of labeled fractions were removed via thoracic duct and portal vein cannulation at 1/4, 1/2, 1, 2, 4, 6 and 8 hours post-injection. Only 1 1/2% of the label was recovered in thoracic duct lymph after 8 hours. Tissue homogenization after sacrifice found 0.6% in the small gut and 5% in a combined fecal-intestinal contents fraction. An apparent 93% portal absorption was inferred by summing the percent recoveries

and subtracting from the 100% administered. The rate of label appearance in the duct was 0.6% in 2 hours and 0.5% by 4 hours, with declines to 0.15% and 0.1% at 6 and 8 hours, resp. In contrast, the rate of appearance in portal blood expressed as disintegrations per min/ml was 75 by 1/4 hour post-treatment, 55 by 1/2 hour, nearly 50 at 1 hour, 60 at 2 hours, 30 at 4 hours and 20 at 8 hours. The second portal maximum at 2 hours probably reflects a combined radioactivity of label already in the circulation and label still being absorbed from the gut.

Column chromatography of ductal lipid fractions found ca. 29% of the 1-C^{14}-caprylic acid label as triglyceride, 2% as minor lipid, 0% as phospholipid or cholesterol ester and 70% as free acid. The percent distribution of label in portal lipid fractions (as a mean of 2 animals) was as follows: phospholipids 0.45, cholesterol ester 0.25, free acid 98.2 and the mono-, di- and triglycerides 0.3, 0.4 and 0.85, resp.

It is concluded that caprylic acid was not esterified effectively by liver enzymes but was removed from circulation by the liver and either catabolized to CO_2 or excreted in the urine.

3. Ref. 134

Carboxyl C^{14}-labeled caprylic acid was given i.v. or p.o. (1.99 mc/mmole) in 2 - 3 μc doses to 16 healthy young adults and 11 patients in fasting condition and symptom-free of liver, pulmonary, heart or lipid metabolic abnormality. The expired $C^{14}O_2$ was monitored continuously for 50 minutes since peak recovery was known to fall between 15 and 30 min.

Recovery of label from 12 healthy subjects was insignificantly different between i.v. (16%) and p.o. (15.5%) administration. The interval between time of administration and metrical evidence of recovery was 1 - 2 min after i.v. and 3 - 6 min after p.o. delivery.

In a second study 4 normal subjects and 6 patients received 2 μc of labeled acid i.v. at 3-day intervals, with a 100g glucose challenge p.o. 3 days later. No difference was found in expired label (22%) over controls (19%), confirming that glucose administration failed to inhibit caprylic acid oxidation.

In a final study 1g unlabeled acid was given i.v. to 5 patients followed by 2 μc labeled acid i.v. The fatty acid load had no effect on amount or rate of caprylic oxidation (20% recovery from each).

The delay period between onset of label recovery after p.o. and i.v. represented the transit from oral cavity to portal circulation. Since there was no portal of entry difference in recovery of expired label, complete intestinal absorption of the tracer dose was inferred.

4. Ref. 41

An infant with chylothorax was subjected to diets with varying fatty acid composition and a relation drawn between dietary and chyle fat. "Capryl olive oil"*, a mixed triglyceride containing 46% caprylic acid wherein each molecule included at least one caprylic group, was given p.o. as 28g daily for 1 week. Pleural aspiration was made weekly. Fat balance and chyle fat content (2.1%) were normal during the treatment period. However, chyle fat contained only 4% of the acid, although the "capryl olive oil" was easily absorbed, and could not be demonstrated in whole blood 3 1/2 hours after administration of the daily dose. It was inferred from the nearly negligible lymphatic absorption that caprylate was converted in liver or intestine and did not pass the liver.

It was also inferred that each fat molecule was almost completely hydrolyzed, since the lymphatic absorption coefficient unique for caprylic acid was only 0.7% (mg acid in chyle/mg acid in diet as percent) and nearly every molecule contained a caprylic group. After hydrolysis, perhaps to a mono-or diglyceride, resynthesis would occur with subsequent transport to chyle, while the caprylic acid molecules liberated would be converted in gut or liver.

*"Capryl olive oil" composition: equal parts olive oil and tricaprylin, mixed and subjected to alkaline reesterification to yield a fat wherein each molecule contained at least one n-caprylic group (to 46% acid-containing): 27.5 mol % tricaprylin, 44.3 mol % "dicapryl olive oil", 23.9 mol % "monocapryl oil", 4 mol % olive oil.

5. Ref. 167 (triglyceride)

For 29 days, 250 - 300 gm Sprague-Dawley rats were fed a nutritionally adequate diet, with 30% of the caloric value being supplied as fat which continued only C_8 and C_{10} acid triglycerides. An end-to-side portacaval anastomosis was then performed on each animal to bypass the liver and they were continued on this diet for another 29 days.

Adipose tissue contained no eight - or ten-carbon fatty acids prior to the experiment; 0.5% of the fatty acid content was octanoate after one month on the diet but prior to portacaval shunt; and 5.4% was octanoate, after two months on the diet and one month after portacaval anastomosis. The increase came principally at the expense of linoleate.

III. Metabolism - Excretion

1. Ref. 49

Two males and 4 females under daily substitution therapy with 0.1-0.15 g dessicated thyroid p.o. were given a suspension of 250 ml whole milk, 20 ml wheat germ oil and 5 μc 1-C^{14}-caprylic acid (sp. ac 2mc/mM) p.o. Since the base line for total exhaled CO_2 in the patients was either normal or slightly depressed, the specific radioactivity in expired air was always elevated. Onset of detectable label usually followed several hours after administration. In patients with tractable primary myxodema the increased level of label returned to normal only very slowly. In contrast to 5 healthy males, specific radioactivity levels in expired air of the 6 patients remained 50% elevated above the normal range after maxima were reached. Whether this deviation was due to the therapy or the disease was not determined.

2. Ref. 163

Fecal assays of 3 healthy male dogs each receiving 454 g of a standard dog food mixed with various fatty acid additives, including 5% caprylic acid daily for 3 successive days elicited the following values of the respective variables, expressed as the range in % of lipids:

	% H_2O	total lipids	Neutral ether extract	soap extract	soap fat (by difference)
standard feed	70-75	17.5-20	3-9	7.5-18	0.4-2.5
5% caprylic acid	70-73	3-7	2.3-4.5	0.8-2.2	10-17.5

The dogs initially refused to eat the standard feed mixed with C_8 acid concentrations above 5%. At the concentration of choice, 5%, the stools were semi-fluid, indicating modest catharsis.

The caprylic acid number of the fecal soap extract was 42.3, the lowest value obtained in the series of fatty acid supplements. Therefore, significant amounts of soap formation - calcium soap was particularly suspected - could not be inferred. The low neutral ether extract and total lipid acid value also denied the presence of large deposits of fecal FFA. It is concluded that the diarrhea and low fecal caprylate follow either from the mixture of small acid doses with large amounts of non-titratable material or else by the intervention of a large molecule with an acid radical.

3. Ref. 55

Jejunal slices from female albino rats (160-200 g) sacrificed after overnight fasting were incubated in bicarbonate buffer at pH 7.4 with 1-C^{14}-caprylic acid (albumin suspension). In a 1-hour assay the recovery of labeled CO_2 from caprylic acid was 200% greater than that from a longer-chain acid (palmitic) or controls. The increment of water-soluble material was also significantly greater (2%) than that from palmitic (0.1%). Label recovery from caprylic acid found 96% in the lipid-soluble fraction (compared to nearly 100% for palmitic). In this fraction the distribution was as follows: monoglyceride and phospholipids 3%, diglyceride 3%, triglyceride 14%, neutral lipid 19% and fatty acid 80%. The fatty acid components were 91% caprylic, 4% C_{10} and 5% C_{12} - C_{20} acids. This 9% of label recovery in long chain acids contrasted with the 96% palmitic acid recovery at C_{16} and confirmed greater gut mucosal catabolism of caprylic acid.

4. Ref. 130

Five Nembutalized male Sprague-Dawley rats, 180-220 g, non-fasted, were injected intraportally with 1.2 μM (2.4 μc) of 1-^{14}C-caprylic acid in 1.0 ml of homogenized albumin - bicarbonate buffer (pH 7.4). Livers were removed 1, 3, 5, 10 and 15 min post-injection.

Liver slices were assayed biochemically and chromatographically after a single intraportal injection of 1.2 μM (2.4 μc) C^{14}- caprylic acid. Very little (12%) of the acid label was incorporated into liver lipid during the 1/4 hour observation. The rate of accumulation of 3-5% for the first 10 minutes to 12% by 15 minutes would suggest a linear increase with time which only repeated passage through the liver would satisfy. However, caprylic acid yielded 5-20% more water-soluble material than longer-chain fatty acids (palmitic), with maxima for both acids reached during the first 60 seconds.

Measured as mμM C^{14}-carboxyl fatty acid incorporated per gram of liver slice in *in vitro* studies, the caprylic/palmitic ratio was 14/307 for lipid-soluble material and 214/15 for CO_2 generation. In the lipid-soluble fraction formed from caprylic acid, triglycerides accounted for 48%, unesterified fatty acids 38% and monoglycerides and phospholipids 10% of the label. Of the unesterified fatty acid

fraction in 2-hour incubations, most of the caprylate underwent elongation: the chain length of more than 6 but less than 16 comprised 17.5%, lengths of 16 ca. 55%, lengths of 18 5% and those between 18 and 20 ca. 23%. Since ca. 80% of the fatty acid label was found among the carboxyl groups, it was likely that the caprylate fraction had been oxidized to acetate or acetyl Co A prior to its incorporation into the long chain unsaturated acids. If the predominant hepatic pathway of oxidation to CO_2 could be applied to extrahepatic systems, caprylate metabolism could be characterized as a fat and carbohydrate sparing agent.

5. Ref. 131

Liver slices prepared from 7-week old male rats (210 g), fed or fasted for 17 hours prior to sacrifice, were incubated with 1-C^{14}-caprylic acid for 2 hours. Measurement of the $C^{14}O_2$ generated in livers from 6 animals lead to mean values of 255 and 127 mμM/g of tissue for the fed and fasted rats resp. Corresponding values of label recovery in lipid-soluble material were 8 and 2. Sex differentiation was insignificant, although there was about 30% cendency toward lower caprylate oxidation with increasing age (from 4 1/2 to 11 weeks). The palmitic acid response was the inverse: little $C^{14}O_2$ yield but high (232-239 mμM) lipid incorporation, a value unaffected by fasting.

Radioactivity in the lipid-soluble fraction was 93% recovered in the fatty acid moieties, confirming an absence of conversion to phospholipid, cholesterol or glyceride glycerol. Some 83% of the recovery was in long-chain acids (length 16 or greater) with less than 2% as original caprylate; more than 1/3 of the longer chain fraction was also desaturated.

In vivo studies (see above study, no. 4, ref. 130) revealed only one sixth as much incorporation of caprylate vs. palmitate. *In vitro* incorporation was of the same order of magnitude, while caprylate oxidation was ca. 6x that of palmitate.

The results of CO_2 production by caprylic acid in other tissues supported the liver data. The values of acid oxidized to $C^{14}O_2$ (in mμM/g tissue) were 700, 350, 350, 160, 200, 70 and 50 in kidney, liver, diaphragm, heart, brain, epididymal fat pad and perirenal fat pad resp. In contrast, the corresponding values for lipid incorporation for the same tissues were 5, 12, 12, 12, 2.5, 11 and 40 mμM/g.

6. Ref. 134

See no. 3, ref. 134 under Absorption-Distribution

7. Ref. 161

Liver slices from 24-hour fasted rats were incubated in carboxyl-C^{13}-labeled sodium caprylate medium at pH 7.4, with subsequent measurement of uptake from one atmosphere of oxygen and the volume of CO_2 liberated. The label was equally distributed between the carboxyl and carbonyl carbon atoms in the acetoacetic acid generated. The result confirmed that fatty acid β-oxidation and subsequent condensation of the 2-carbon intermediates formed (probably acetic acid) were responsible for ketogenesis. However, only ca. 20% of the caprylic acid incubated could be identified with ketone body formation and 4 - 5% by oxidation to CO_2. Moreover, oxygen consumption was very significantly lower than calculations of complete acid oxidation would predict. The presence of unknown non-oxidative pathways for caprylic catabolism is suggested.

8. Ref. 83

Growing Wistar rats (less than 8 months old) were fed either normal or steatogenous diets (20% lipid and 8% protein) for 1-5 months and then sacrificed. Droplets of fat were observed in the cytoplasm and nucleoplasm of liver cells, accompanied by hypertrophy of Golgi apparatus in those animals fed a steatogenous diet. The fat content of the liver approached six times normal in those rats maintained on this diet for the full five months.

The isolated liver mitochondria were incubated with buffered octanoate, also containing ATP, salts and cytochrome c. Octanoate was oxidized almost exclusively to acetoacetate, and only a negligible quantity of CO_2 was formed. The rate of production of acetoacetate per gram of mitochondrial nitrogen by mitochondria from animals fed a steatogenons diet was equivalent to or higher than those from normal hepatic tissue.

9. Ref. 110

The ability of liver slices from four month old hooded rats to catabolize octanoate was examined. One group each of males and females was castrated six weeks prior to sacrifice. Others, instead, were given hormone treatment i.v. for 10 days prior to sacrifice - 5 mg daily of tesosterone to the males and 1 mg daily of estradiol to the females.

Neither castration, in both sexes, nor testosterone treatment of males had any effect on the ability of liver slices to convert octanoate to acetoacetate or CO_2. However, liver from estradiol treated females showed a 42% drop in octanoate to acetacetate conversion.

IV. Effects on enzymes and other biochemical parameters

1. Ref. 144

Standard (I) and steatogenous (II) diets were given to 22-day old male Wistar rats for 28 days and liver homogenates were assayed for C_8-oxidase activity. II was free of choline and vitamin B_{12}. The difference in oxygen consumption between experimental and control values in 20 min for I and II was 1.3 and 0.87 μM/mg. N, resp., while the values for caprylate disappearance were 0.86 and 0.74. Oxygen consumption values for diet II represented a 30% decrease in C_8-oxidase activity. The ratio of extra oxygen consumption to caprylate disappearance was 1.5 and 1.1 for I and II, resp.

Additional studies related caprylate metabolism to ketogenesis. After diet I, 1.12 μM/mg N caprylate disappeared, and 0.95 μM/mg N ketone bodies appeared in 20 mins; after diet II, the values were 0.81 and 0.65. The ratio of ketone formation to caprylate disappearance for I and II was 0.85 and 0.8, resp.

The data would suggest that upwards of 75% of the disappearing caprylate is ketogenic, with the fate of the remainder unknown, and that caprylate oxidation in liver is inhibited by high fat, low protein diets.

2. Ref. 77

Sodium caprylate at various molarities was added to 110 mg ovalbumin in phosphate buffer and the tryptic digestion coefficient measured spectrophotometrically at 5, 10, 20 and 40 mins. An unheated preparation of 0.1 or 0.15M C_8 acid potentiated tryptic digestion of ovalbumin to approximately the same degree as preparations heated to 80 or 98 degrees F. At 0.8M caprylate significantly inhibited both the unheated and heated preparations. At 0.05M the caprylate effect on native protein simulated that of trypsin alone on unheated preparations.

Polarimetry found no change in optical rotation of ovalbumin solution at 0.05M. However, there was a shift to levo at 0.1M, confirming direct denaturation of ovalbumin by C_8 acid.

3. Ref. 60

Hemidiaphragms from Sprague-Dawley rats fed either fat or carbohydrate diets before sacrifice were paired into control and caprylic acid incubation systems at pH 7.4 for 2 hours. The carbohydrate tissue removed more caprylate than the fat tissue at an anion concentration of 2 μM per ml; at 3.3 μM there was no difference in anion uptake. Both dose levels of caprylate stimulated oxygen uptake 14% for both diets. This result was confirmed by animals on stock diets at 0.5 and 1.0 μM anion concentrations. However, final concentrations from 3.0 to 5.0 μM per ml tended to inhibit oxygen consumption toward the latter part of the 2-hour incubation during which interval tissue uptake of caprylate was maximal and a detergent effect may have been responsible for the inhibition.

4. Ref. 112 (triglyceride)

A medium chain triglyceride (1000 mg/kg) consisting of 80% caprylate, 17% decanoate and traces of hexanoate and laurate was given to 10 men and 4 women on 2 separate mornings 1 week apart. The serum ketone average rose from 0.77 to 6.7 mg/100 ml at 2 hours and remained above 6 mg for 5 hours. Serum glucose fell 10 mg/100 ml (from ca. 90 mg/100 ml) during the first hour and remained at plateau (ca. 85 mg/100 ml) for 5 hours. Serum insulin rose from 9.3 to 17.7 μU/ml at 1/2 hour, falling to 15.3 at 1 hour and 12 at 4 and 5 hours. Since a predominantly C_8 triglyceride elicited significantly greater increase in serum insulin than long chain triglycerides (corn oil), the effect can not be related to release of fat-mediated gastrointestinal hormone. Nor is it known whether the modest hyperinsulinemia followed from the hyperketonemia or the escape of some caprylate from complete hepatic catabolism.

5. Ref. 45

A 4 cm segment of fresh rabbit jejunum was exposed to a 0.5-0.75 mg % minimal concentration sodium caprylate in glucose-free Krebs-Henseleit solution and aerated with 95% O_2 - 5% CO_2 for maintenance of pH 7.4. Kymographic assay showed that caprylate elicited a 40-60% restoration of gut contraction amplitude, based on 100% rebound on glucose addition to a glucose-free medium in which motility had been completely suppressed. Caprylate-induced recovery was equivalent

to that for C_4 and C_6 acids, while odd-numbered chains in the same range had zero to limited effectiveness. Once an amplitude maximum was reached, for each acid separately, the effect after combined acid administration was non-additive. A 1.5 mg % caprylate concentration also restored the 80% inhibition by propionic acid (30 mg %) during caprylate-induced (0.75 mg %) contractions, thus confirming the prediction of a non-metabolizable propionic residue after β-oxidation of odd-carbon fatty acids. Glyceraldehyde suppression of motility was also ca. 50% compensated by caprylate.

Relatively high concentrations of caprylic acid (25-150 mg%) led to a 100% inhibition of motility. The effect was reversible. It is assumed that degree of contraction maintenance varies directly with the magnitude of energy an agent supplies for smooth muscle contraction.

6. Ref. 53

In violation of the theory that lipid solubility of fatty acids increases with carbon chain length, both the extent and rate of caprylic acid penetration in bovine and human erythrocytes was found to be lower than that for C-5, but higher than those for C_1 - C_3 acids. Both spectrophotometry (measuring oxygen loss) and parpart densimetry (measuring hemolysis) were confirmatory. It is inferred that membrane penetration is not in general an independent result of erythrocyte lipid solubility. Molecular size above the C_6 length was thought to be a limiting factor in the rate of penetration: lipid solubility would be decisive for C_1 - C_3 and molecular size for C_6 - C_8. The compromise values obtained for C_4 and C_5 would represent a cancellation effect between lipid solubility and molecular size.

7. Ref. 160

Fresh auricles from young rabbits were incubated in Tyrode's solution perfused by 95% O_2 - 5% CO_2 for maintenance of pH 8.1. Concentrations of 2×10^{-5} M caprylate at 0.5, 1, 3 and 5 minute exposures failed to alter the contraction rate, but there were 4 and 9% inhibitions of amplutide at longer intervals. A 6% rate inhibition followed 10^{-4} M after 3 and 5, and 5×10^{-4} M after 1/2

and 5 minutes exposure. Higher rate depressions were achieved by 5×10^{-4} M at 1 minute (17%), 10^{-3} M for 2 minutes (14%) and 10^{-2} M at both 1 and 2 minutes (22%). Increasing amplitude reduction was obtained by 10^{-4} M at 3 and 5 minutes (16 and 26%, resp.), 5×10^{-4} M at 1 and 5 minutes (11 and 24%, resp.), 10^{-3} M at 1 and 2 minutes (15 and 25%, resp.) and 10^{-2} M at 1 and 2 minutes (33 and 42%, resp.). Recovery from caprylate inhibition simulated that from citrate and, unlike lower chain anions, was impossible pending complete washing from medium.

8. Ref. 135

Heparinized platelet-rich rabbit plasma failed to release significant histamine or serotinin after incubation with 50-250 μg/ml caprylic acid, a characteristic of unsaturated or lower molecular weight saturated fatty acids.

9. Ref. 82

Caprylate (1.6 mM) was 50% inhibitory of hexokinase activity in hepatoma 3924-A incubation, while a 2.9 mM concentration was required for the same effect in normal rat liver. Glucokinase activity in normal rat liver was 50% inhibited by 0.4 mM caprylate; tumor glucokinase was not detectable. A 50% inhibition of renal cortex and renal tumor (9789-k) hexokinase was reached by 1.7mM and 2.3mM octanoate, resp. However, glycolytic enzyme inhibition was considerably enhanced by the longer-chain acids, which would indicate a considerably higher carcinolytic index than that of caprylic acid.

10. Ref. 89

Insulin secretion was assayed during incubation of islets of Langerhans from fasted male albino Wistar rats with sodium caprylate. Both insulin liberation and intracellular glucose-6-phosphate increased 100% when 5.0 mM caprylate was added to 2.5 mM glucose in the medium, an effect roughly equivalent to 20 mM glucose alone.

Short-chain acids are said to affect the accumulation of intracellular citrate with subsequent inhibition of phosphofructokinase, inhibition of glycolysis and glucose-6-phosphate accumulation. Since glycolysis is inhibited by fatty acids, the data suggest that sugar-induced insulin release is not mediated by glycolysis. Some other

pathway of glucose catabolism would be required to stimulate insulin secretion (a "glucose fatty acid cycle").

11. Ref. 105

Isolated rat hearts were perfused with various caprylate concentrations in a medium at pH 7.4 and cardiac contractility monitored by intraventrical balloon. The mean control values were 50 cm (H_2O) for peak systolic pressure (PSP) and 214 beats per minute for heart rate. A 0.175 mM caprylate solution reduced PSP by 30% and subsequent increases in concentration up to 2.8 mM diminished the PSP an additional 10% within 2 minutes. Neither 0.175, 0.35 nor 0.7 mM affected heart rate, but 1.4 mM reduced it slightly and 2.8 mM led to a 20% fall. Arrhythmias were recorded only at 2.8 mM (3:1 A-V block), with some evidence of quadrigeminy after protracted perfusion with 1.4 mM. Reversal effects followed glucose re-exposure.

The rate of glucose oxidation was depressed to 11% of the control when 2.8 mM sodium caprylate was added to 5 mM glucose; no arrhythmias were detected.

12. Ref. 65

Administration of buffered 0.5 M caprylic acid i.v. in cats induced spindles and slow waves in the cruciate EEG after 3 doses of 1 ml each. Cortical blood flow increased after 6 doses, while the typical initial arousal reaction on reticular formation stimulation waned after subsequent stimuli. Pressor responses also declined. High plasma glucose failed to change the neural depression. The whole neurovascular response pattern simulated that of physiological sleep.

Paralleling the "humoral" effect of short-chain acids (butyric), the caprylate anion is thought to suppress cortical activation by the mesencephalic reticular system.

13. Ref. 137

Spectrophotometric evidence is presented confirming that fatty acids compete with substrate for occupancy of hydrophobic binding sites of enzyme and react with the enzyme via their lipophilic termini. The degree of competitive inhibition was found to increase with aliphatic chain length. The inhibition constant (Ki) for 5×10^{-3} M caprylic acid was 2.1×10^{-3} M for N-acetyl-L-tyrosine ethyl esterase systems.

14. Ref. 91

Blood ketone (up 3.5 mg % from a normal 2) and glucose levels (down 20 mg % from a normal 90) remained unremarkable in a dog receiving 35 ml of isotonic sodium caprylate solution i.v. (jugular or saphenous) one week before fasting and 30 ml more 5 days into a 17 day fast. Five days before terminating the fast 32 ml i.v. generated a transient 100% increase (up to 12 mg %) in ketonemia (within 20 min.), with a tendency toward hypoglycemia. Four days later 29 ml i.v. elicited only a 4 mg % rise in ketone body formation but discrete hypoglycemia. Weight loss during the period was 2 kg. One day post-fasting the animal received a fattening diet and 30 ml caprylate i.v. There was only a 3 mg % Ketonemia (from 3 mg %) for 10 minutes, but it remained elevated at 5 mg % indefinitely. The hypoglycemia amounted to 40 mg % during the first 30 min. Two weeks later 33 ml acid elicited transient insignificant Ketonemia but the fall in blood glucose remained equally marked. Body weight was restored to 8.4 kg. Standard protein feeding was renewed 10 days later. Subsequent caprylate i.v. loads of 30 ml each at 1 and 3 weeks elicited peaks of 4 - 5 mg %, along with an equally sharp hypoglycemia of 30 - 40 mg %; rapid normalization followed. These results were confirmed in other dog experiments.

Cheyne-Stokes respiration was an occasional concomitant of i.v. administration. Some animals were disoriented and appeared "benumbed" up to 10 min post-injection. Adaptation followed after several caprylate challenges.

15. Ref. 155

A solution containing 3 mg Na octanoate and 5 mg pepsin per l. was acidified. The liberated octanoic acid was adsorbed by the pepsin and thus inhibited by 21% its activity toward oval bumin. This effect was less than that with longer chain fatty acids.

V. Drug effects

1. Ref. 86

A 500 ml solution of 1.5% sodium caprylate was infused i.v. for 1 hour (a total of ca. 100 mg/kg) in 14 mildly ill patients between metabolic disease between 23 and 58 years of age without metabolic disease, either combined with 20 g glucose or 20 g glucose plus 0.1 unit/kg body weight HGF-fill insulin. Blood was drawn via both femoral artery and hepatic venous catheters and ketogenesis assayed.

Net splanchic ketogenesis (NSK) was a mean 106 μM/min/meter^2 (a 33% decrease from controls), with 9% of the caprylate converted to ketone bodies in the glucose series. NSK was 125 μM/min/meter^2, a 25-30% decrease over controls, with 11% of the caprylate converted to ketone bodies in the glucose-insulin series. Although the percent of caprylic ketogenesis was roughly equivalent in both series, arterial blood ketone levels were generally lower in the glucose-insulin group. In a 6-subject study of acute transient insulin hypoglycemia the 3-hour posthypoglycemic NSK reached 266 μM/min/meter^2, a nearly 65% rise over controls. This potentially high posthypoglycemic ketogenesis elicited by exogenous caprylate would suggest either an accelerated rate of liver caprylate consumption or increased release from adipose tissue and delivery to liver for ketogenesis.

Since carbohydrate sparing of intermediate chain oxidation was assumed to be minimal, the net glucose effect was evidently an interruption of intracellular caprylate oxidation and not a reduction in tissue utilization in ketone bodies.

2. Ref. 24

Male Wistar rats weighing 150-250 g were sacrificed after *ad lib* feeding and the diaphragms or hemidiaphragms dissected for incubation in Gey's medium with caprylate and labeled leucine (0.05 mM) with or without insulin or glucose. In cut muscle preparations (2 hemidiaphragms dissected and incubated separately),

3mM caprylate reduced C^{14} incorporation from leucine into protein by 12% in the presence of 1 mg/ml glucose. In the presence of 1 mU/ml insulin (which increased label incorporation from 800 to 1205 mμ moles/g protein) and 1 mg/ml glucose, caprylate decreased C^{14} by 32%. The result with intact diaphragm and 1mM acid showed insulin-induced protein reduced by 27%, but only 13% without insulin. With intact hemidissected diaphragm (a careful midline cut for paired experiments) and insulin but no glucose, 1mM caprylate diminished incorporation by 23%; without insulin or glucose the loss was 15.5%.

A 0.5 mM caprylic and insulin incubation with glucose and intact hemidissected muscle reduced label incorporation 17%; increasing the fatty acid concentration to 1mM in the same system led to a 24% reduction.

A 10-fold increase in leucine concentration to 0.5mM in the same system elicited a 28% drop, confirming that caprylate itself inhibits protein synthesis and is not dependent on isotope dilution effects of the amino acid source.

C^{14} uptake from an insulin-free medium with 0.05mM leucine and 1 mg/ml glucose led to a 40% higher hemidiaphragmatic utilization of label with 1mM caprylate. C^{14} oxidation also rose 200%. Uptake was not affected by insulin addition, but caprylate potentiated insulin-induced C^{14} oxidation by 170% and elicited a 110% increase in oxidation over insulin-free controls.

Using 3-C^{14}-α-aminoisobutyric acid (AIB), a non-utilizable amino acid, as index of muscle cell penetration, small but significant increases in sorbitol and tissue water distribution occurred at concentrations of 3mM, but not 1mM, caprylate. Since amino acid label uptake from the medium was higher, but label incorporation into protein lower, during caprylate incubation, amino acid metabolism must have been altered after the membrane transport phase. However, the AIB data would suggest membrane perturbation by high caprylate concentrations. Insulin stimulation of AIB transport was also depressed by the higher concentration.

3. Ref. 32

Male mice of C57/Fn strain and 3 - 5 months old received either a 60% fat or carbohydrate diet for 2 weeks prior to the administration of a pituitary growth hormone preparation (10 mg/kg body weight), with sacrifice 5 hours later. Livers were subsequently incubated with caprylate in medium B, such that each ml of medium contained the mitochondria from 1g of liver, 0.5 μc of C^{14}-leucine and 1mg of synthetic amino acid mixture. On the high fat diet there were ca. 280 and 294 counts/min/mg protein/hour C^{14}-leucine incorporation, resp., with and without growth hormone. For high carbohydrate diets the values were 287 and 171 counts, resp., with and without hormone. Thus, mitochondrial incorporation on caprylate substrate remained significantly unaltered by growth hormone in animals with dietary fat, but not carbohydrate, adaptation. The rate of protein synthesis was also conspicuously elevated in fat-adapted hormone-free environments. The data suggests that a time-dependent fat adaptation can serve as surrogate for growth hormone in eliciting energy for protein synthesis via a more rapid lipid catabolism during carbohydrate depletion.

4. Ref. 84

An aqueous solution containing glucose, sodium taurocholate, sodium chloride and caprylic acid (139,20,8.5 and 10 mg/ml, resp.) was infused in the proximal duodenum of 4 healthy and 5 cirrhotic human subjects, all without diabetic history. A total of 360 ml were administered over one hour (ca. 50 mg/kg total caprylic acid). Serum chromatography found a 2-fold increase in glucose fasting levels during glucose infusion alone in all of the normal persons, but no effect with caprylic-glucose, a pattern characteristic also of the hepatic patients. A rise in fasting serum insulin (immunoreactive) after glucose alone was significantly elevated by caprylate addition for both healthy and diseased subjects. Serum total FFA fell in both groups after glucose alone, while caprylic acid with glucose had no effect on the rate of fall.

Serum caprylate was detectable within 10 minutes following combined infusion in both groups, with peak concentrations occurring by 1/2 hour. Normalization of caprylate levels on termination of infusion was slower in the cirrhotic group. Serum caprylic levels in 2 additional cirrhotic patients receiving intraduodenal acid only simulated the levels in patients receiving the glucose-caprylic combination; no glucose or insulin modification was detected.

The evidence that, (a) serum insulin rose only during caprylate absorption; (b) caprylic acid failed to modify liver glucogenesis and (c) serum fatty acid was insignificantly elevated by caprylic infusion would suggest either a direct pancreatic effect or an indirect effect based on the release of an unknown insulin-inducing factor in the gut wall during caprylate absorption *in situ*.

5. Ref. 159

Male and female mongrel dogs (12-20 kg) were anesthetized and treated with deoxycorticosterone. For 60 min. prior to and during the experiments they were subjected to i.v. injection of a solution containing 120mM NaCl, 24mM $NaHCO_3$ and 3mM KCl, at a rate of 1ml/min. Enough creatine and p-aminohippurate (PAH) were given simultaneously to maintain the plasma levels near 8-12 and 1- 2.5 mg/100ml, respectively.

After ureter catherization sodium octanoate in salt-serum albumin solution was injected into one renal artery at rates of 0.38 - 1.94ml/min for 25 min. At doses of 39 μ mole/min there was no ipsilateral effect on natriuresis, renal plasma clearance of creatine or tubular or total clearance of PAH. At slightly higher dose levels local arterial plasma levels of octanoate increased by 0.5 - 1.8mM. At doses of 86-94 μm/min a reduced total clearance of PAH was noted. This reduction was mediated by large decreases (33-60%) in tubular clearance of PAH, but no noticeable decrease in renal plasma clearance of creatine, glomerular filtration rate or sodium diuresis was observed.

VI. Consumer Exposure Information

Data was provided by the NAS/NRC comprehensive GRAS survey, Oct., 1972, Tables 2 and 13A.

Food category	No. firms reporting	usual use (mean ppm)	max. use (mean ppm)
Baked goods baking mixes	13	9.1	25.5
Fats and oils	*	4.0	27.5
Frozen dairy desserts mixes	6	1.1	4.6
Meat products	*	3.7	7.1
Soft candy	8	3.6	7.0
Gelatins, puddings	*	5.0	14.3
Snack foods (except nuts and popped corn)	*	41.0	82.0
Beverages, non-alcoholic (except milk and fruit juices)	8	0.6	1.1
Beverages, alcoholic and cocktail mixes	*	3.0	**
Gravies, sauces	*	2.0	4.0
Imitation dairy products	*	4.0	8.0
Hard candy	*	4.5	4.5
Chewing gum	*	0	8.6
Misc. unclassified	*	35.4	203.5

* indicates that 3 or fewer firms reported

** indicates that the level supplied by the respondents was considered to be incorrectly reported and could not be reconciled

Food Categ.	Firms Reporting	Age of consumer	Possible daily intake(mg)		
			Avg.	High A	High B
Baked goods	13	0-5 mos.	0.03	0.04	0.09
		6-11 "	.23	.47	.65
		12-23 "	.50	.82	1.39
		2-65+ yrs	1.25	1.85	3.50
Fats and oils	*	as	0.00	0.00	0.01
		above	.01	.03	.08
			.03	.05	.17
			.07	.13	.48
Frozen dairy	6		0.00	0.00	0.00
		as	.01	.03	.04
		above	.02	.04	.07
			.03	.13	.12
Meat prod	*		0.00	0.01	0.01
		as	.08	.21	.15
		above	.11	.19	.21
			.29	.48	.56
Soft candy	8		0.00	0.01	0.00
		as	.01	.02	.02
		above	.01	.03	.02
			.02	.06	.04
Gelatin, puddings	*		0.01	0.01	0.03
			.06	.19	.18
		as	.07	.17	.20
		above	.10	.26	.29
Snack foods	*		----	0.00	----
		as	.02	.05	.03
		above	.05	.13	.09
			.05	.15	.11
Beverages, non-alc.	8		0.00	0.00	0.00
		as	.01	.05	.02
		above	.03	.10	.06
			.06	.17	.11
Beverages, alc.	*		0.00	0.00	**
		as	----	.00	**
		above	----	.00	**
			.10	.28	**

Gravies	*		0.00	0.00	0.00
		as	.00	.01	.01
		above	.01	.02	.01
			.02	.04	.03
Imit. dairy	*		0.00	0.00	0.00
		as	.01	.01	.01
		above	.00	.01	.01
			.00	.01	.01
Hard candy	*		0.00	0.00	0.00
		as	.00	.00	.00
		above	.00	.00	.00
			.00	.01	.00
All categories	21		0.05	0.09	0.15
		as	.44	1.07	1.19
		above	.82	1.56	2.24
			2.00	3.51	5.25

Values in this table are rounded to the nearest 0.01 mg and are based on data from the Market Research Corp. of America on frequency of eating of foods and the USDA data on mean size of food portions.

Avg. - usual use/mean consumption

High A - usual use/high consumption

High B - maximum use/mean consumption

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Appendix A

to

CAPRYLIC ACID

A Monograph

Prepared for

Department of Health, Education and Welfare

Public Health Service

Food and Drug Administration

Rockville, Maryland

by

Dr. Paul C. Walter and

Dr. Steven J. Love

FIRL Project No. 80G-C3378-01

December 14, 1972

THE FRANKLIN INSTITUTE RESEARCH LABORATORIES

CAPRYLIC ACID

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[Agr. Biol. Chem. Vol. 30, No. 9, p. 863~868, 1966]

Studies on Flavor Components in Soybean

Part III. Volatile Fatty Acids and Volatile Amines*

5

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Department of Agricultural Chemistry, The University of Tokyo, Tokyo

Received February 26, 1966

Both acidic and basic fraction of flavor components in raw soybean were investigated. The acidic fraction, having a weak but characteristic odor, contained nine sorts of volatile fatty acids, which were identified with gas chromatography as acetic, propionic, isovaleric, *n*-valeric, isocaproic, *n*-caproic, *n*-caprylic, *n*-nonanoic (pelargonic), and *n*-capric acid. Among those, main components were isocaproic, *n*-caproic and *n*-caprylic acid, and the others were contained in smaller amounts. Total amount of these acids, however, was so small that it would have little practical effect on soybean flavor.

The basic fraction exhibited a characteristic odor just like a dried fishery product, and ammonia, monomethylamine, dimethylamine, piperidine, and cadaverine were detected in this fraction with gas and paper chromatography. Particularly, dimethylamine was isolated as 2,4-dinitrophenyl derivative.

INTRODUCTION

Soybean has a peculiar, unfavorable flavor which prevents the positive utilization for foodstuff. The authors have reported the presence of several sorts of aliphatic carbonyl compounds and phenolic acids as flavor components of soybean.[1,2] In relation to odor of soybean, it was expected that both acidic and basic components contributed to it. Concerning the acidic components, H. M. Teeter et al.[3] has reported the presence of several sorts of fatty acids in defatted soybean flour, the main component of which was *n*-caproic acid. On the other hand, S. Sasaki[4] has reported the existence of cadaverine as a basic substance in etiolated soybean seedlings. But any other reports have not been known in these connections. The authors studied on volatile components in both acidic and basic fraction of raw soybean.

MATERIALS

Soybean. Raw soybean (Hawkeye, 1964) was used.

Authentic samples. Various volatile fatty acids of C_2 to C_{10} were obtained from commercial sources and volatile amines such as ammonia, monomethylamine, dimethylamine, piperidine (hexahydropyridine), and cadaverine (pentamethylenediamine) were also obtained from commercial sources.

EXPERIMENTAL

Investigation of volatile fatty acids.

Preparation of sample for gas chromatography. The raw soybean was meaked just before the use. Two hundred g. of the metal which passed through 16 mesh sieve was extracted with 500 ml of dichloromethane for 5 hours using a large Soxhlet's apparatus. This solvent has been applied by I. R. Hunter to

* Presented at the Annual Meeting of the Agricultural Chemical Socity of Japan, Kyoto, April 4, 1965.

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4) S. Sasaki, *J. Dept. Agr., Kyushu Imp. Univ.*, 5, 51 (1936).

extract fatty acids in a fermentation liquor.[5] The dichloromethane solution was shaken with equal volume of 0.1 N sodium hydroxide in order to transfer acidic components from the organic layer to the aqueous layer. To the aqueous layer separated was added 85% phosphoric acid and the pH was adjusted to 8.0. The neutralized solution was evaporated to dryness at room temperature under reduced pressure. The white powder obtained, a mixture of sodium salts of acidic components, was acidified with small volume of 2 N sulfuric acid, and then 10 ml of ether was added to extract fatty acids. A small amount of dried sodium sulfate was added to eliminate the water. The resulting ether solution was directly submitted to gas chromatography.

Gas chromatography for volatile fatty acids. Many reports have been presented on gas chromatographic analysis of volatile fatty acids from various sources.[6~10] As it is a recent tendency that the fatty acids are analysed without being esterified, the authors adopted this procedure. The liquid phase consisted of silicone-stearic acid mixture was applied here for the direct analysis which has been reported by C. L. Hankinson et al.[6] A Shimadzu Model GC-1C gas chromatographic unit, employing a thermal conductivity detector, was used. The detector cell was operated [illegible]0°C. The U-shaped column of 3 mm in inside [illegible]ter and 140 cm long was packed with Silicone DC-550 (95 parts) containing stearic acid (5 parts) (Shimadzu Seisakusho, Ltd.). The column temperature was 90°C for fatty acids lower than C_6 and 140°C for those higher than C_6. A preheater was maintained at 300°C. Carrier gas (helium) flow rate was 35 ml per minute. The sample mentioned above was applied directly and the retention times of the peaks were compared with those of authentic samples. Furthermore, the odor was checked up organoleptically at the outlet point of the gas chromatographic unit whenever each peak appeared.

Investigation of volatile amines.

Preparation of sample for paper chromatography. Two hundred g. of the raw soybean was dipped in 2 liter of 10% trichloroacetic acid solution for 10 hours at room temperature. The swollen soybean was crushed and stirred in this soluiton for 5 hours. The trichloroacetic acid solution is a very convenient solvent for extracting basic components in soybean because it does not dissolve fatty materials and protein. R. B. Hughes[11] has used this solvent for extracting volatile amines in herring flesh. The extract was filtered and the residue was washed with small volume of the solvent. The filtrate was neutralized to pH 5 with sodium hydroxide, mixed with equal volume of ethanol, and allowed to stand overnight in refrigerator. The resulting white precipitate was filtered off and the filtrate was concentrated at room temperature under reduced pressure until almost all the ethanol was distilled out. The residue (about 500 ml), after being washed repeatedly with ether to eliminate residual fatty materials completely, was adjusted to pH 8.0 with sodio phosphate and steam-distilled for 30 minutes. The distillate was trapped in a bottle containing 200 ml of 2 N hydrochloric acid. The resulting hydrochloric acid solution was washed with equal volume of ether and concentrated to dryness at rom temperature under reduced pressure. The white powder obtained, a mixture of hydrochloric acid salts of volatile amines, was submitted to paper chromatography.

Paper chromatography for volatile amines. Two kinds of solvent systems, *n*-butanol : ethanol : water (4 : 1 : 1 by vol.) and *n*-butanol : acetic acid : water (4 : 1 : 5 by vol.), were used. Experimental conditions were as follows: Toyo Filter-paper No. 51, ascending method, and room temperature. Four kinds of color reagents were used. Ninhydrin reagent (0.1% in *n*-butanol), Nestler reagent and iodine vapor were prepared by usual methods. Dragendorff reagent was prepared according to the method of R. Munier.[12]

Gas chromatography for volatile amines. The above-mentioned gas chromatographic unit was used. The detector cell was operated at 200°C. The U-shaped column of 3 mm in inside diameter and 140 cm long was packed with triethalamine (5 parts) on Diasolid A (80- to 100-mesh) (95 parts) (Nippon Chromato Kogyo, Ltd.). The column temperature was 50°C. A preheater was maintained at 200°C. Carrier gas (helium) flow rate was 36 ml per minute. The hydrochloric acid salts of volatile amines prepared for paper chromatography were treated with dilute solu-

5) I. R. Hunter, Hawkins, NG, and J. W. Pence, *Anal. Chem.*, 32, 1757 (1960).
6) C. L. Hankinson, W. J. Harper, and E. Mikolajcik, *J. Dairy Sci.*, 41, 1502 (1958).
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11) R. B. Hughes, *Nature*, 181, 1231 (1958).
12) R. Munier, *Bull. Soc. Chim. Biol.*, 35, 1225 (1953).

tion of sodium phosphate
stopper to be adjusted to
water for one minute. T
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Preparation of 2,4-di
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King,[13] by which amines

Peak No.	Corresp Fatty
1	acetic
2	propi
3	isoval
4	*n*-vale
5	isocap
6	*n*-cap
7	*n*-cap
8	*n*-non
9	*n*-cap

13) A. M. Asatoor, *J. Chro*
14) L. Ekladius and H. K.

tion of sodium phosphate in a small test tube with a stopper to be adjusted to pH 8.0 and heated in boiling water for one minute. The gas phase in this tube was directly injected to the gas chromatographic unit using a usual syringe. Furthermore, the liquid phase was shaken with small volume of ether and the ether layer was submitted to gas chromatography. The retention times of the peaks obtained were compared with those of authentic samples and the organoleptic tests were carried out by the method similar to the one described previously.

Preparation of 2,4-dinitrophenyl derivatives of volatile amines. The hydrochloric acid salts of volatile amines were treated with dilute solution of sodium phosphate to be adjusted to pH 8.0 and the resulting free amines were extracted with ether. To the ether extract was added ethanol solution of 1-chloro-2,4-dinitrobenzene. As ether was easily evaporated off, only an yellow-colored ethanol solution of 2,4-dinitrophenyl (2,4-DNP) derivatives of amines was obtained, which was submitted to paper chromatography according to the method of A. M. Asatoor.[13] The spot corresponding to 2,4-DNP derivative of authentic dimethylamine was cut off from the paper chromatograms and these fragments were treated with ethanol. To the ethanol extract was added water little by little until white turbidity appeared. After allowing to stand overnight, yellow needle crystal was obtained, which was submitted to infrared spectrometry.

Determination of total amount of volatile amine. Total amount of volatile amine was determined by measuring the color intensity of 2,4-DNP derivative solution using the method of L. Ekladius and H. K. King,[14] by which amines could be determined without any interference of ammonia.

RESULTS AND DISCUSSION

Volatile fatty acids.

As shown in Fig. 1 and Table I, nine sorts of volatile fatty acids were detected. Among those, the main acids were isocaproic, *n*-caproic and *n*-caprylic acid, and the others existed in considerably small amounts. Total

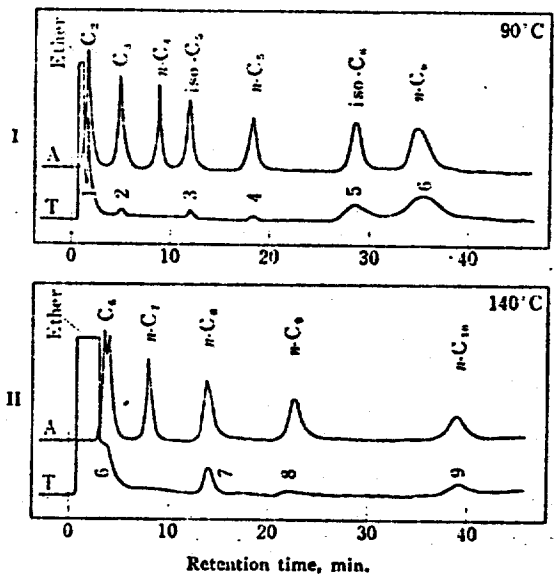

FIG. 1. Gas Chromatograms of Volatile Fatty Acids
A: Authentic samples; T: Test samples

TABLE I. CHARACTERIZATION OF EACH PEAK

Peak No.	Corresponding Fatty Acid	Retention Time (min.) I	Retention Time (min.) II	Flavor Characteristic	Amount
1	acetic	2.8	—	acetic acid like	++
2	propionic	5.0	—	—	+
3	isovaleric	12.0	—	—	+
4	*n*-valeric	18.4	—	—	+
5	isocaproic	28.8	4.0	green bean like	++
6	*n*-caproic	36.0	4.0	green bean like	+++
7	*n*-caprylic	—	14	green bean like	++
8	*n*-nonanoic	—	23	waxy	+
9	*n*-capric	—	40	waxy or butter like	+

13) A. M. Asatoor, *J. Chromatog.*, 4, 111 (1960).
14) L. Ekladius and H. K. King., *Biochem. J.*, 65, 128 (1957).

amount of the volatile fatty acids in the raw soybean was so small, although the accurate determination was not carried out, that it seemed to have little practical effect on soybean flavor, but the concentrated acidic fraction had obviously fatty acid-like odor, to which these three acids would make main contributions.

Volatile amines.

As shown in Fig. 2 and Table II, several sorts of volatile amines were detected with paper chromatography. Among those, the spots 1, 2, 3 and 5 were identical in R_F-values with those of piperidine, dimethylamine, monomethylamine and cadaverine, respectively. The spot 4 proved to be ammonia because Nestler reaction was positive. Fig. 3, the chromatograms of 2,4-DNP derivatives, showed the presence of ammonia, monomethylamine, dimethylamine and piperidine. Furthermore, gas chromatograms of Fig. 4 also showed the presence of these compounds. Total amount of volatile amine in the raw soybean was approximately 1.5 p.p.m., the greater part of which was considered, according to Fig. 4, to be dimethylamine. The identification of this compound was carried out with infrared spectrometry in comparison with authentic dimethylamine, the result being shown in Fig. 5.

Considering from these results, the dried fishery product flavor of the raw soybean may be mainly due to dimethylamine, and piperidine, having a characteristic flavor, may have some minor effect on this flavor. Cadaverine, also having a flavor similar to that of piperidine, was actually detected as shown in Fig. 2, but it was irreproducible, the reason of which was not investigated. It is considered that volatile amines are produced occasionally

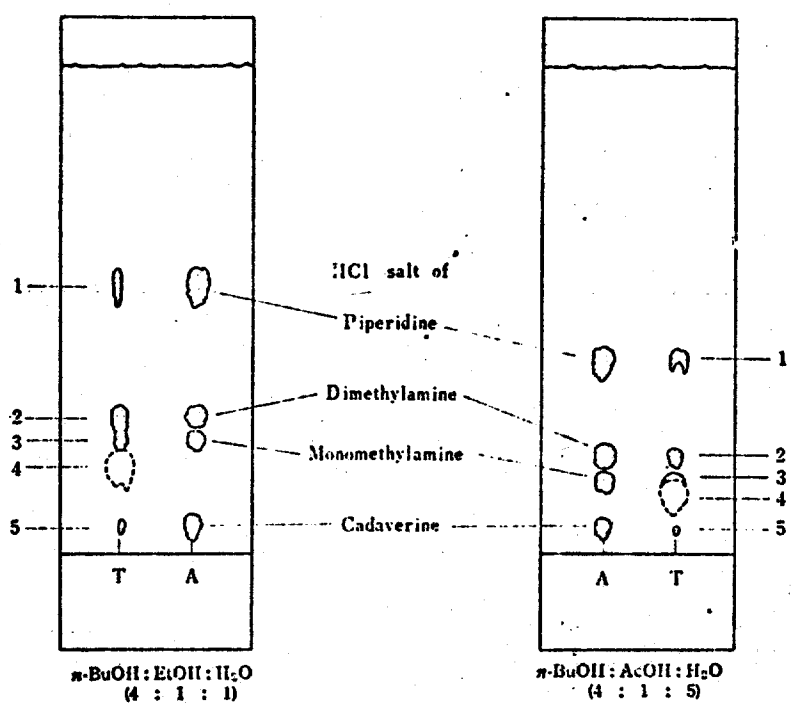

FIG. 2. Paper Chromatograms of Amine Hydrochlorides

A: Authentic samples; T: Test samples

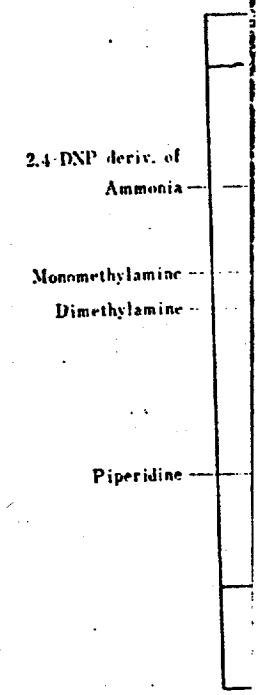

FIG. 3. Paper Chrom
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A: Authentic samples
Solvent: Upper layer of a
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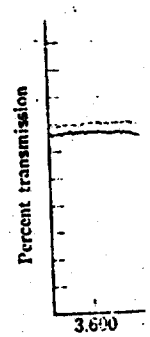

piperidine. Furthermore, of Fig. 4 also showed the npounds. Total amount of raw soybean was approxi- he greater part of which nding to Fig. 4, to be di- identification of this com- ut with infrared spectro- with authentic dimethyl- ng shown in Fig. 5.

these results, the dried of the raw soybean may nethylamine, and piperi- teristic flavor, may have this flavor. Cadaverine, imilar to that of piperi- ected as shown in Fig. 2, ble, the reason of which

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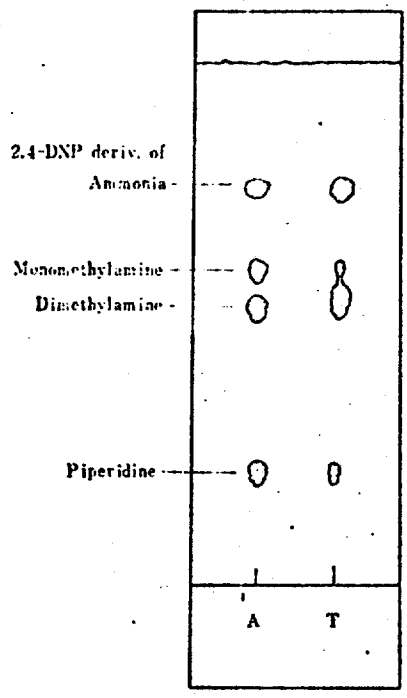

FIG. 3. Paper Chromatograms of 2,4-DNP derivs. of Amines

A: Authentic samples; T: Test samples
Solvent: Upper layer of a mixture of $CHCl_3$:MeOH:H_2O (10:10:6);
Filter paper: Toyo Filter-paper No. 51 impregnated in petroleum ether containing 10% of liquid paraffin.

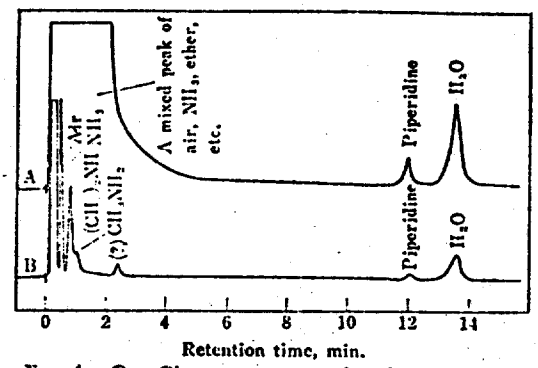

FIG. 4. Gas Chromatograms of Volatile Amines
A: Ether solution; B: Vapor

TABLE II. COLOR REACTIONS OF EACH SPOT

Spot No.	Dragendorff Reaction	Ninhydrin Reaction	Iodine Reaction	Nestler Reaction
1	brown red	+	+	−
2	brown red	+	+	−
3	brown red	+	+	−
4	—	+	−	+
5	blue violet	+	+	−

through decarboxylation of corresponding amino acids during heat treatment of foods, but probably this reaction did not occur in the experimental conditions applied here, the conclusive evidence of this fact having been reported by C. Weurman and Corry de Rooij.[15]

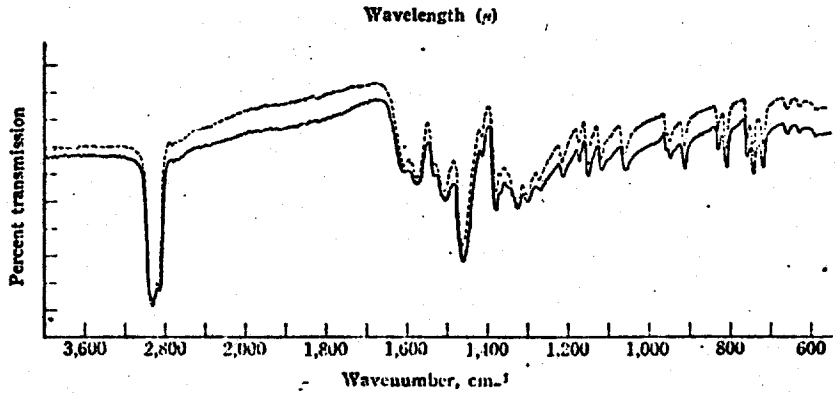

FIG. 5. Infrared Spectra (Nujol)

Upper: authentic 2,4-DNP deriv. of dimethylamine; Lower: sample

15) C. Weurman and Corry de Rooij, *J. Food Sci.*, 26, 239 (1961).

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Part 1. Fluo

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The Oxidative Metabolic Pattern of Mouse Hepatoma C_{954} as Studied with C^{14}-labeled Acetates, Propionate, Octanoate, and Glucose*

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Weinhouse and co-workers (39–43) have established that the Krebs tricarboxylic acid cycle operates in a variety of experimental tumors and that fatty acid oxidation, believed to be nonexistent in tumors (9), provides 2-carbon fragments for continuous operation of this cycle in neoplastic tissues. Our studies with the mouse hepatoma C_{954}, carried by mice of the C57-Leaden strain, also led us to conclude that the oxidation of octanoate by tumor tissue followed the same course as in normal tissues (4, 8). The labeling of acetoacetate, formed after incubation of tumor slices with octanoate-1-C^{14}, was consistent with the mechanism of β-oxidation-cleavage-condensation observed in normal tissues (4, 8).

The present experiments were undertaken to determine whether any well defined derangement existed in the metabolism of a neoplastic tissue. Comparisons were made of normal mouse liver, host liver, and hepatoma C_{954}, all of which were allowed to oxidize radioactive substrates *in vitro*. Acetate-1-C^{14}, acetate-2-C^{14}, octanoate-1-C^{14}, propionate-1-C^{14}, pyruvate-2-C^{14}, and uniformly labeled glucose were employed as substrates. Incorporation of C^{14} from the substrates into various intermediates was studied by a chromatographic-autoradiographic technic. The present experiments with hepatoma C_{954} fully confirm, in general, the observations of Weinhouse and co-workers (39–42) and extend those observations to include, in the metabolic spectrum of tumor tissue, several reactions peripheral to the tricarboxylic acid cycle. Most reactions observed in the normal liver proceed in the hepatoma, although certain differences do exist.

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MATERIALS AND METHODS

Labeled substrates.—Sodium acetate-1-C^{14} and -2-C^{14} and sodium propionate-1-C^{14} were synthesized in this laboratory by Dr. S. Abraham, by conventional methods. Sodium octanoate-1-C^{14} was kindly supplied by Dr. W. G. Dauben of the Department of Chemistry. Dr. Abraham also prepared uniformly-labeled glucose (2) photosynthetically by the method of Putman and Hassid (31). Pyruvate-2-C^{14} was purchased from Isotope Specialties, Inc.

Buffers.—Krebs-Ringer phosphate and Krebs-Henseleit bicarbonate buffers were prepared as described by Umbreit *et al.* (34).

Animals and their tissues.—The hepatocarcinoma C_{954} carried by mice of the C57-Leaden strain was originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. We are indebted to Mr. Carl Emanuel for maintaining this tumor by serial subcutaneous transplantation. Tumors and livers were removed about 2 weeks after implantation of the hepatoma. (The mice rarely survive more than 3½ weeks after implantation.) The tumors selected in this study always appeared to be free of necrosis.

In vitro procedures.—Slices of normal liver, hepatoma, or host liver were prepared either free-hand or with the aid of a McIlwain-Buddle (26) automatic tissue slicing machine (Mickle Co.). Whenever the tumor tissue was not solid enough to slice, the loose tissue was merely suspended in the incubation medium. Flasks containing center wells for collection of respiratory CO_2 were employed. The tops of the flasks were closed with rubber caps (12) through which acid could be injected to stop reactions. Pertinent details of the incubation in the several experiments are given in the tables.

Analytical procedures.—Respiratory CO_2, collected in the alkaline center wells of the flasks, was precipitated as $BaCO_3$ and assayed for C^{14} (18). Aqueous extracts of the reaction mixture were examined by the chromatographic-autoradiographic technic (3) as applied to animal tissues (17). After the extracts were desalted electrolytically (10), they were subjected to two dimensional chromatography on Whatman No. 4 filter paper sheets (18 × 22 in.). The solvents employed were phenol saturated with water, in the short dimension, and n-butanol-acetic acid-water (50:11:22) in the long dimension. Autoradiograms were prepared by placing the air-dried paper chromatograms in contact with 14 × 17-inch "No-Screen" x-ray films (Eastman Kodak Co.). Radioactive areas of the chromatograms were outlined and were assayed for C^{14} with a 2.5-inch diameter, thin end-window Geiger tube (3). Amino acid areas were detected or verified with ninhydrin, glucose

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with aniline-trichloroacetic acid (or tetrazolium chloride), and acid areas with bromcresol green. Spots obtained on the autoradiograms and/or chromatograms were identified by reference to standard chromatographic maps. Intermediates were identified by their relative positions, rather than by their R_F's in the solvents (3).

Where applicable, aliquots of aqueous extracts were steam-distilled in the presence of H_2SO_4. Acetoacetate is lost in this step. The volatile fraction obtained corresponds to unreacted, volatile, isotopic substrates (acetate, octanoate, propionate), while the nonvolatile fraction corresponds to those compounds appearing on the paper chromatograms. (When glucose was employed as substrate, no distillation was carried out.) Aliquots of the acid-volatile and nonvolatile fractions were made basic and counted directly on aluminum planchets. Appropriate mass correction factors were used whenever necessary.

The incorporation of isotope into each compound resolved by the chromatographic-autoradiographic method was first

TABLE 1

INCORPORATION OF ISOTOPE FROM OCTANOATE-1-C^{14} INTO VARIOUS FRACTIONS BY NORMAL AND HOST LIVER AND BY HEPATOMA C_{88} OF MICE

(Phosphate Buffer)

(0.50 gm. of slices [wet wt.] incubated for 2 hours in 5.0 ml. Ca^{++}-free Krebs-phosphate buffer, pH 7.4, 37.3° C.; oxygen as gas phase, alkali in center well. Concentration of octanoate, 0.001 M; 542,000 counts/min incubated per flask. Reactions stopped with HCl + heat. Experiments with one normal mouse and one tumor-bearing animal.)

Tissue	Per cent of incubated C^{14} recovered as: Respired CO_2	Nonvolatile fraction	Volatile fraction	Total
Normal liver	48.4	32.5	12.8	93.7
Hepatoma	38.7	22.5	2.4	63.6
Host liver	49.4	36.7	7.3	93.4

expressed as a fraction or percentage of the total counts/min found on the chromatograms. The per cent of the incubated counts/min incorporated into any compound was then given by the expression: per cent of incubated counts/min found in nonvolatile fraction × (counts/min in compound on paper divided by total counts/min on paper).

It was observed that unreacted octanoate-1-C^{14} was not completely volatilized from the chromatograms. Therefore, in expressing the percentage of the total counts/min recovered in any compound on the chromatograms, the C^{14} of the area corresponding to isotopic octanoate was not included in the total counts/min.

The per cent of incubated counts/min found for any compound is independent of the total counts/min on the paper chromatograms. Values in the tables usually represent the average percentages obtained from several chromatograms. The reproducibility which may be attained is illustrated by the two chromatograms of Table 8.

Acetoacetate was determined by the acid-heat decarboxylation of the buffer solution in the presence of mercuric sulfate (35). Carrier acetoacetate was added before decarboxylation.

When pyruvate-2-C^{14} was chromatographed in the two solvent systems, it yielded at least six different radioactive areas on the autoradiograms, a major one of which corresponded to that of pyruvate. Decomposition of the pyruvate probably took place during storage and not during the chromatographic procedure (cf. 27). The results of the experiments with the radioactive pyruvate are therefore not unequivocal, but it was possible to determine whether or not glucose had been formed. The results of the experiments with pyruvate are not presented in detail because of this complication.

RESULTS AND DISCUSSION

PRELIMINARY STUDIES WITH MOUSE HEPATOMA C_{88}, NORMAL MOUSE LIVER, AND HOST LIVER OCTANOATE-1-C^{14}

Slices of host liver, normal liver, or hepatoma were incubated in 5 ml. of calcium-free Krebs-Ringer phosphate buffer containing 5 μM of octanoate-1-C^{14}. Details of the incubation and of the incorporation of isotope into respired CO_2, the nonvolatile fraction, and the volatile fraction appear in Table 1. About one-half the incubated radioactivity was recovered in CO_2 in the case of normal and host liver, and somewhat less (about 40 per cent) for the hepatoma. About one-third of the isotope appeared in compounds of the nonvolatile fraction in the case of normal and host liver, and somewhat less (about 20 per cent) for the hepatoma. As judged by the counts/min recovered in the volatile fraction, very little of the substrate remained after the 2-hour incubation period.

Photographs of the autoradiograms for the three tissues are presented in Figures 1, 2, and 3. As many as a dozen radioactive areas are discernible. Compounds that contained C^{14} are listed in Table 2 as percentages both of the nonvolatile and of the incubated counts/min. These are arranged in descending order of isotope incorporation for normal liver. (See "Materials and Methods" for determination of the per cent of incubated counts/min incorporated into a given compound.)

Practically all the isotope on the chromatograms from the three tissues resided in the same compounds, although differences in percentage incorporation did exist. In keeping with what we noted earlier (19), not much isotope was present in members of the tricarboxylic acid cycle, but C^{14} was found in compounds closely associated with acids of that cycle. Thus glutamate and aspartate are amino acids derived from the cycle acids, α-ketoglutarate and oxalacetate, respectively. Glucose, alanine, and lactate are compounds derived from pyruvate, the latter known to be produced from oxalacetate. β-Hydroxybutyrate, on the other hand, arises by reduction of acetoacetate formed by condensation of 2-carbon fragments derived from octanoate. Urea is formed by fixation of CO_2 into citrulline (21). Pyrrolidone carboxylic acid, a cyclic derivative of glutamic acid, is presumed (17) to arise, nonenzymatically for the most part, before or during chromatography. It is possibly an intermediate in the formation of proline from glutamate (32).

The high incorporation of isotope into glutamic acid and the low incorporation into glutamine for hepatoma are quite striking as compared with normal or host liver. As judged by the intensity of

the glutamate and glutamine areas after spraying with ninhydrin, these two amino acids were present on the chromatograms in approximately the same amount. It seems that the rate of conversion of glutamate to glutamine is limited in the hepatoma.

The hepatoma seemed to have little capacity to form *radioactive* glucose, although detectable quantities of glucose (as revealed by spray reagents) were present on the paper chromatograms. This glucose may have been derived from the blood stream. It is thus conceivable that, even *in vivo*, the hepatoma synthesizes little or no glucose. This point requires further study. In this connection it should be noted that Zamecnik *et al.* (46) also found that gluconeogenesis (from pyruvate-2-C^{14}) in a rat hepatoma was much less than in slices of normal liver.

In this preliminary experiment the percentage of isotope recovered in glucose in the case of normal liver, while higher than that for hepatoma, was anomalously low.

A Comparison of the Oxidation of Acetate-1-C^{14} and Octanoate-1-C^{14} in Hepatoma and Host Liver Slices

According to reports from other laboratories (5, 30), acetate appears to be poorly activated to the reactive metabolic 2-carbon fragment (acetyl-S(CoA) by neoplastic tissues. However, the short-chain fatty acids, butyrate and octanoate, were shown to be readily converted to CO_2 and acetoacetate by the mouse hepatoma C_{254} (8). In the preliminary experiments reported above it was shown that the isotope of octanoate-1-C^{14} was incorporated into intermediates by the hepatoma in a fashion similar to that of normal or host liver. It was, therefore, of interest to extend these observations to see how acetate compared with octanoate in its ability to label various intermediates. The experimental protocol and the results are presented in Table 3.

In both liver and hepatoma, octanoate-1-C^{14} was utilized in preference to acetate as judged by (*a*) conversion to $C^{14}O_2$, (*b*) incorporation into the

TABLE 2

Incorporation of Isotope from Octanoate-1-C^{14} into Nonvolatile Compounds

(Corresponds to experiments reported in Table 1)

Compounds	Normal liver: Per cent of nonvolatile counts/min	Normal liver: Per cent of incubated counts/min	Hepatoma C_{44}: Per cent of nonvolatile counts/min	Hepatoma C_{44}: Per cent of incubated counts/min	Host liver: Per cent of nonvolatile counts/min	Host liver: Per cent of incubated counts/min
β-Hydroxybutyrate	58.5	19.0	31.7	7.2	73.1	26.8
Glutamate	14.1	4.6	40.3	9.1	2.6	1.0
Glutamine	6.7	2.2	0.8	0.2	6.3	2.3
Di- and tri-COOH acids	6.0	2.0	12.5	2.8	4.1	1.5
Aspartate	3.3	1.1	3.5	0.8	2.1	0.8
Glucose	3.2	1.0	1.3	0.3	6.9	2.5
Alanine	2.2	0.7	4.6	1.0	1.9	0.7
Pyrrolidone-COOH acid	1.2	0.4				
Lactate	1.1	0.4	1.5	0.3	1.0	0.4
Urea	1.1	0.4			1.3	0.5
Others (unidentified)	1.1	0.4				
Glutathione	0.8	0.3	3.5	0.8		
Origin	0.8	0.3	0.7	0.2	0.3	0.1

TABLE 3

Incorporation of Isotope from Acetate-1-C^{14} and Octanoate-1-C^{14} into Various Fractions by Host Liver and Hepatoma C_{44} of Mice

(Bicarbonate Buffer)

(0.50 gm. slices [wet wt.] incubated for 3 hours in 5.0 ml. Krebs-Henseleit bicarbonate buffer, pH 7.4, 37° C.; 95 per cent O_2-5 per cent CO_2 as gas phase. Concentration of acetate, 0.008 M; of octanoate, 0.002 M. Reactions stopped with HCl + heat. Tissues from several animals were pooled.)

Tissue and Substrate	Recovered CO_2	Per cent of incubated C^{14} recovered as: Acetoacetate*	Nonvolatile fraction	Volatile fraction	Total
Liver:					
Acetate-1-C^{14}	8.8	1.1	4.9	73	88
Octanoate-1-C^{14}	20.5	16.5	43.0	24	104
Acetate-1-C^{14}+octanoate	3.1	0.67	3.3	77	84
Hepatoma:					
Acetate-1-C^{14}	3.3	0.15	1.4	79	84
Octanoate-1-C^{14}	18.0	1.9	7.3	77	104
Acetate-1-C^{14}+octanoate	1.4	0.10	0.7	80	82

* Determined from flasks incubated under conditions identical with those from which the nonvolatile and volatile fractions were analyzed. Carrier acetoacetic acid was added before analysis.

nonvolatile fraction, (*c*) incorporation into individual intermediary products, and (*d*) conversion to acetoacetate. The molar concentration of the labeled acetate incubated was 4 times that of the octanoate, so that an equivalent number of potential 2-carbon fragments were represented. When nonisotopic octanoate was incubated with acetate-1-C^{14}, an inhibition in the conversion of the isotope to CO_2, nonvolatile fraction, intermediary products, and acetoacetate was observed for both liver and hepatoma.

It is clear from the data of Table 3 that the utilization of acetate for CO_2, acetoacetate, and nonvolatile fraction is less in the hepatoma than in liver. It may be calculated that labeled CO_2 was produced from the substrate at the rate of 39 mμM × (gm. wet wt. × min.)$^{-1}$ by host liver, but at only 15 mμM × (gm. wet wt. × min.)$^{-1}$ by the hepatoma.

The nonvolatile compounds in the experiments of Table 3 were examined, and the data are summarized in Table 4. In general, the *relative* distribution of isotope in the various compounds of the nonvolatile residue was the same with acetate-1-C^{14} and octanoate-1-C^{14}. These compounds have been arranged arbitrarily in three groups according to the percentages of isotope (with respect to the total C^{14} in all compounds found on the paper chromatograms). The three arbitrary groups are: major incorporation products, >5 per cent; intermediate incorporation products, 1–5 per cent; and minor incorporation products, <1 per cent; these values represent percentages of isotope of the nonvolatile residues.

Host liver.—Glutamine, β-OH-butyrate, glucose, and glutamate were *major* incorporation products for acetate-1-C^{14}, octanoate-1-C^{14}, and for acetate-1-C^{14} in the presence of nonisotopic octanoate (Table 4). While β-hydroxybutyrate (22.2 per cent) and glutamine (23.4 per cent) were about the same when derived from acetate-1-C^{14}, a marked difference in distribution of isotope was observed when β-hydroxybutyrate (57.9 per cent) and glutamine (12.0 per cent) were derived from octanoate-1-C^{14}. Decrease of isotope from octanoate-1-C^{14} in glutamine and most compounds (except β-hydroxybutyrate) was a consequence of the greater ketogenic activity of octanoate as compared with acetate.

Intermediate incorporation products in host liver were lactate, alanine, and urea. The di- and tricarboxylic acids (taken as a group) showed some variability.

Minor incorporation products were glutathione, aspartate, and unidentified compounds at the origin. Since there was no evidence of appreciable amounts of radioactive glycine or cysteine, the glutathione probably contained only the labeled amino acid residue, glutamate.

When acetate-1-C^{14} was incubated with nonisotopic octanoate, the distribution of C^{14} among the compounds on the chromatogram did not resemble that when acetate-1-C^{14} alone or octanoate-1-C^{14} alone was incubated. The conclusion may be drawn that 2-carbon fragments derived from acetate-1-C^{14} interacted with those derived from nonisotopic octanoate, and the C^{14} thereby assumed a metabolic pattern somewhere between that of the two labeled substrates.

It can be noted that, when acetate-1-C^{14} was incubated with nonisotopic octanoate, acetate participated in the ketogenic activity of octanoate.

TABLE 4

INCORPORATION OF ACETATE-1-C^{14} AND OCTANOATE-1-C^{14} INTO NONVOLATILE COMPOUNDS

(Corresponds to experiments of Table 3. Figures in parentheses denote percentages of nonvolatile residue. Average values for two or more chromatograms are reported.*)

Incorporation products	Host liver slices			Hepatoma slices		
	Acetate-1-C^{14}	Octanoate-1-C^{14}	Acetate-1-C^{14} +octanoate	Acetate-1-C^{14}	Octanoate-1-C^{14}	Acetate-1-C^{14} +octanoate
Major: >5 per cent of nonvolatile counts/min	Glutamine (23.4) β-Hydroxybutyrate (22.2) Glucose (18.6) Glutamate (10.2) Pyrrolidone-COOH acid (8.6)	β-Hydroxybutyrate (57.9) Glutamate (12.0) Glucose (7.7) Glutamate (7.0) Di- and tri-COOH acids (5.7)	β-Hydroxybutyrate (12.1) Glutamine (13.4) Glucose (13.2) Glutamate (8.7) Di- and tri-COOH acids (7.8)	Glutamate (10.0) Di- and tri-COOH acids (12.5) Lactate (12.2) Glutamine (10.2) β-Hydroxybutyrate (9.1) Aspartate (6.8)	Glutamate (33.7) Di- and tri-COOH acids (10.0) β-Hydroxybutyrate (12.4) Lactate (11.9) Aspartate (6.9) Glutamine (5.0)	Glutamate (33.1) Di- and tri-COOH acids (23.3) Lactate (14.9) β-Hydroxybutyrate (13.7) Glutamine (7.0)
Intermediate: 1–5 per cent of nonvolatile counts/min	Lactate (4.7) Di- and tri-COOH acids (3.2) Alanine (2.7) Urea (1.4)	Lactate (2.3) Pyrrolidone-COOH acid (1.7) Alanine (1.4) Urea (1.1)	Lactate (4.1) Pyrrolidone-COOH acid (3.9) Alanine (2.6) Urea (2.0)	Pyrrolidone-COOH acid (3.7) Alanine (3.0) Glutathione (2.9) Origin (1.3) Glucose (1.2)	Alanine (4.9) Pyrrolidone-COOH acid (4.2) Origin (3.4) Glutathione (3.0)	Glutathione (2.8)
Minor: <1 per cent of nonvolatile counts/min	Glutathione (0.9) Aspartate (0.7) Origin (0.3)	Glutathione (0.4) Origin (0.3) Aspartate (trace)	Origin (1.0)			

* Percentages of isotope incorporation do not necessarily add up to 100 per cent, since they represent average values.

This special property of octanoate may be traced to the terminal-type 2-carbon fragment (11) peculiar to fatty acids of the even series. The different patterns for acetate and octanoate are traceable, in some measure, to the predilection of this 2-carbon fragment for the carbonyl moiety of acetoacetate and of β-hydroxybutyrate. The reactivities of the 2-carbon fragments of acetate and fatty acids are discussed at length elsewhere (7, 11, 24).

Hepatoma C_{964}.—The incorporation of the C^{14} of acetate-1-C^{14} into CO_2, acetoacetate, and nonvolatile compounds by the hepatoma was less than one-third that observed for host liver (Table 3). β-Hydroxybutyrate was the predominant radioactive compound in host liver (as was glutamine in experiments with acetate-1-C^{14}), but the major incorporation products with tumor from octanoate-1-C^{14} were, in generally decreasing order: glutamate, di- and tricarboxylic acids (taken as a group), β-hydroxybutyrate, lactate, aspartate, glutamine. A few inversions in this order occurred with acetate-1-C^{14}, but the six compounds were nevertheless members of the major incorporation products. Intermediate incorporation products observed in tumor experiments with octanoate-1-C^{14} were alanine, glutathione, and the origin.

Several features of the metabolic pattern of the hepatoma deserve comment. The experiments with octanoate-1-C^{14} will be discussed here, although the comments also apply generally to experiments with acetate-1-C^{14}. In the experiment under consideration ketogenesis was greater in the host liver than in the hepatoma as judged by acetoacetate (Table 3) or β-hydroxybutyrate formation (Table 4).

The data of Table 4 emphasize that (aside from β-hydroxybutyrate, glutamine, and glucose) all compounds in the hepatoma experiment contained greater percentages of the nonvolatile C^{14} than was the case with host liver. Quite striking is the difference in the value for the glutamate-C^{14}/glutamine-C^{14} ratio. This ratio had a value of 0.58 in host liver, but a value of 6.75 in hepatoma as a result of both an increased isotope incorporation into glutamate and a decreased incorporation into glutamine. Note that glutamate accounted for one-third of the isotope of the nonvolatile fraction in the case of hepatoma.

Another metabolic shift found in the hepatoma was evidenced by the relatively larger amounts of isotope incorporated into the di- and tricarboxylic acids. While these acids constituted a borderline case in the major incorporation products for host liver, they represented, in the hepatoma, 19 per cent of the nonvolatile residue. The relative incorporation of isotope into lactate, aspartate, alanine, and glutathione was also higher, presumably at the expense of isotope in β-hydroxybutyrate (Table 4).

In the experiment with host liver appreciable amounts of isotope were incorporated into glucose. In the case of hepatoma, glucose again contained little isotope (see above). Nonincorporation of isotope in glucose does not seem to be the result of a failure to deliver isotope from cycle intermediates to pyruvate or phospho-*enol*-pyruvate, because both lactate and alanine contained isotope. To establish further that pyruvate-C^{14} contributed negligibly to glucose-C^{14} formation, pyruvate-2-C^{14} was incubated with hepatoma and host liver slices. The hepatoma formed radioactive lactic acid, but yielded little, if any, glucose. Host liver, on the other hand, did convert pyruvate-2-C^{14} to glucose as well as to lactate.

TABLE 5

INCORPORATION OF ISOTOPE FROM OCTANOATE-1-C^{14} INTO VARIOUS FRACTIONS BY HOST LIVER AND HEPATOMA C_{964} OF MICE

(Phosphate Buffer)

(0.5 gm. of slices [wet wt.] of liver or tissue dispersion of hepatoma, incubated for 2 hours in Ca^{++}-free Krebs-Ringer phosphate buffer, pH 7.5, 37.5° C. Oxygen as gas phase, alkali in center well. Concentration of octanoate, 0.002 M; 1.97×10^6 counts/min incubated per flask. Four animals employed. Reactions stopped with HCl + heat.)

Tissue	Per cent of incubated C^{14} recovered as: Respired CO_2	Nonvolatile fraction	Volatile fraction	Total
Host liver	40.0	22.3	2.6	64.9
Host liver	34.7	24.4	5.6	64.7
Hepatoma	34.0	16.0	13.1	63.1
Hepatoma	26.6	14.1	19.3	60.0

The utilization of octanoate-1-C^{14} by hepatoma, observed in experiments reported in this section, was lower than that reported in the previous section (Table 1). The preliminary experiments were carried out in a phosphate buffer instead of with a bicarbonate buffer. It seemed desirable to determine whether a difference in buffer could account for the observed difference in utilization.

Incubation of host liver and hepatoma slices in phosphate buffer.—As judged by the isotope in the volatile fraction (unreacted substrate), in respired CO_2, and in the nonvolatile fraction, utilization of octanoate-1-C^{14} by hepatoma in phosphate buffer (Table 5) again exceeded that observed in bicarbonate buffer (Table 3). The incorporation of isotope into the nonvolatile residue was higher in phosphate than in bicarbonate buffer for hepatoma, while the reverse was true for host liver. In the experiments shown in Table 5, an appreciable

fraction (about 35 per cent) is not accounted for. This isotope may have resided mostly in acetoacetate, which was not analyzed.

Compounds of the nonvolatile residue that contained C^{14} are presented in Table 6. Characteristically, the conversion of isotope to β-hydroxybutyrate, for hepatoma, was higher with phosphate than with bicarbonate buffer. The metabolic pattern observed with hepatoma incubated in the bicarbonate buffer held when this tumor was incubated in the phosphate buffer—for example, high glutamate-C^{14}/glutamine-C^{14} ratio, small amount of isotope in glucose, and elevated aspartate-C^{14} and di- and tricarboxylic acid isotope levels. Variations observed from experiment to experiment, whether due to buffer, nutritional state, substrate concentrations, or age of the hepatoma, seemed not to affect the general, over-all metabolic picture.

TABLE 6

INCORPORATION PRODUCTS FROM OCTANOATE-1-C^{14}

(Correspends to experiments of Table 5. Figures in parentheses denote percentage of nonvolatile residue.)

Incorporation products	Host liver	Hepatoma
Major: >5 per cent of nonvolatile counts/min	β-hydroxybutyrate (45.4) Glutamate (11.5) Glucose (7.6) Glutamine (6.9) Lactate (5.7)	Glutamate (36.4) β-hydroxybutyrate (28.2) Di- and tri-COOH acids (8.8) Lactate (6.6) Aspartate (5.3)
Intermediate: 1–5 per cent of nonvolatile counts/min	Urea (4.4) Pyrrolidone-COOH acid (3.5) Alanine (3.1) Di- and tri-COOH acids (2.2) Glutathione (2.0) Aspartate (2.0)	Glutathione (3.9) Pyrrolidone-COOH acid (2.8) Alanine (2.7) Origin (1.8) Glutamine (1.6) Urea? (1.0)
Minor: <1 per cent of nonvolatile counts/min	Origin (0.8)	

OTHER ASPECTS OF ACETATE OXIDATION BY HEPATOMA

Participation of the tricarboxylic acid cycle.—Presumptive evidence that the tricarboxylic acid cycle operates in the hepatoma was provided above by (*a*) oxidation of labeled 2-carbon fragments (generated from acetate or octanoate) to $C^{14}O_2$; (*b*) incorporation of isotope from 2-carbon fragments into compounds of the cycle and to other compounds (glutamate, aspartate, alanine); and (*c*) a pattern of isotope labeling similar to that of liver, in which the cycle is known to operate. Additional evidence was provided by finding that malonate, a competitive inhibitor of succinate oxidation, inhibited the conversion of acetate-1-C^{14} to $C^{14}O_2$ by a suspension of the hepatoma.

Rate of oxidation of acetate to CO_2.—Bush and Baltrush (5, 6) found that, when acetate-1-C^{14} was injected into rats bearing neoplasms, from 70 to 100 per cent of the total isotope reaching most tissues was rapidly converted into nonvolatile compounds. In the case of tumor tissues, the bulk of the isotope resided in the volatile portion, i.e., acetic acid. These workers concluded that tumors are largely inert in their behavior toward acetate *in vivo*. It is to be noted that, previously, Pardee, Heidelberger, and Potter (30) found that slices of two transplantable tumors exhibited only a slight ability to convert acetate-1-C^{14} to $C^{14}O_2$, as compared with kidney and liver.

TABLE 7

FATTY ACID INHIBITION OF ACETATE OXIDATION IN HEPATOMA

(0.83 gm. of a cell suspension of hepatoma incubated for 3 hours in 5.0 ml. Krebs-Henseleit bicarbonate buffer, pH 7.5, 37.5° C.; 95 per cent O_2–5 per cent CO_2 as gas phase. Concentration of substrates: Na acetate-1-C^{14}, 0.008 M; butyrate, hexanoate, octanoate, and decanoate, 0.002 M; lauric acid, 0.001 M. All fatty acids were neutralized before being added to flasks. Tumor slices from three mice were pooled.)

Fatty acid added with acetate-1-C^{14}	Per cent inhibition*
None	0
Butyrate	77
Hexanoate	70
Octanoate	70
Decanoate	67
Laurate	70
None	74
	0

$$* \left(1 - \frac{\text{Per cent } C^{14}O_2}{\text{Per cent } C^{14}O_2 \text{ in absence of acid}}\right) \times 100.$$

We also found a low rate of acetate oxidation by mouse hepatoma. It is not enigmatic that acetate is poorly utilized while octanoate is readily oxidized, for these substrates are activated by different enzymes (15). Poor utilization of acetate by this as well as by other hepatomas (5, 30) may not be a general property of tumors, for other workers have found the utilization to be about the same as or higher than (29) that of normal liver.

Inhibition of acetate-1-C^{14} by short-chain fatty acids.—A reduction in the utilization of acetate-1-C^{14} by hepatoma C_{254} in the presence of octanoate was observed above (Table 3). Octanoate was found by Lehninger (23), Walkenstein and Weinhouse (37), and by Vestling *et al.* (36) to inhibit markedly the tricarboxylic acid cycle and to decrease oxygen uptake in slices or homoge-

nates of normal tissues. The effectiveness of octanoate and other short-chain fatty acids in inhibiting the oxidation of acetate-1-C^{14} by the mouse hepatoma was tested. The experimental conditions and results appear in Table 7. In the presence of either butyrate, hexanoate, octanoate, decanoate, or laurate, the conversion to $C^{14}O_2$ was inhibited about 70 per cent. Similarly, the incorporation of isotope into the nonvolatile residue was depressed by the short-chain fatty acids. If the depression in $C^{14}O_2$ production were due merely to dilution of isotopic 2-carbon fragments by nonisotopic fragments generated from the short-chain fatty acids, one would expect the $C^{14}O_2$ production to vary with the chain length of the fatty acids (assuming each was converted to 2-carbon fragments at the same rate). But the percentage inhibition was remarkably constant (see Table 7). The results point to an actual depression in the activation of acetate by the short-chain fatty acids. This may be owing to competition for activation as the acylSCoA. If, however, the short-chain fatty acids do limit the oxidative disposal of 2-carbon fragments from acetate by inhibiting some step in the tricarboxylic acid cycle, then the rapid utilization of the short-chain fatty acids themselves remains unexplained.

Utilization of acetate-2-C^{14} by mouse hepatoma.—The dynamics of the tricarboxylic acid cycle are such that the methyl carbon of a 2-carbon fragment is incorporated into intermediates of the cycle to a greater extent than is the carboxyl carbon (19, 33). This leads to a greater incorporation of the acetate methyl carbon into glycolytic intermediates, since these may be derived from acetate only after the latter has entered the cycle.

The pattern of labeling of intermediates from acetate-2-C^{14} by hepatoma was studied and compared with that obtained from a carboxyl-labeled, 2-carbon fragment (e.g., acetate-1-C^{14} or octanoate-1-C^{14}). It was anticipated that denser radioactive areas on the autoradiograms would be obtained with acetate-2-C^{14} at comparable levels of isotope in the incubation medium. The chromatograms were to be examined specifically for the presence of radioactive glycine (see below).

Table 8 summarizes the data on the isotope incorporation into various compounds. A typical autoradiogram of a chromatogram from which the data of Table 8 were compiled appears as Figure 4. In the present experiment, 3.9 per cent of the isotope appeared in the nonvolatile components, and 3.0 per cent appeared in $C^{14}O_2$.

The major incorporation products with acetate-2-C^{14} were glutamate, lactate, alanine, aspartate, di- and tricarboxylic acids (as a group), and β-hydroxybutyrate. The principal difference noted in the present experiment, as compared with the experiment in which acetate-1-C^{14} was employed, is that alanine and lactate contain much more isotope. While 30 per cent of the counts/min on the chromatograms resided in lactate + alanine, again little or no isotope was found in glucose. Only 1.3 per cent of the nonvolatile counts/min resided in the area occupied by glucose, glycine, and serine. Because of the low percentage incorporation into this area, it was not feasible to determine which of these three compounds contained the activity.

The area corresponding to citrate was eluted and rechromatographed one-dimensionally in 1-

TABLE 8

CONVERSION OF METHYL CARBON OF ACETATE TO VARIOUS METABOLITES BY HEPATOMA C_{70}

(0.50 gm. of tumor dispersion [wet wt.] incubated for 3 hours in 5.0 ml. Krebs-Henseleit bicarbonate buffer, pH 7, 37° C.; 95 per cent O_2–5 per cent CO_2 as gas phase. Concentration of acetate-2-C^{14}, 0.004 M. 2.64 × 10^7 counts/min incubated in each flask. Tumors from three mice were pooled.)

Compound	Per cent of total C^{14} on chromatogram	
	Paper 1	Paper 2
Glutamate	30.5	28.2
Lactate	17.3	19.5
Alanine	11.0	11.6
Di- and tri-COOH acids	10.4	11.7
Aspartate	11.0	10.1
β-Hydroxybutyrate	6.2	6.0
Urea?	2.9	2.5
Orlcin	2.9	1.8
Glutathione		2.8
Glutamine	2.2	1.9
Peptide?	1.6	1.2
Pyrrolidone-COOH acid	1.4	1.0
Glucose	4.3	1.3
Unidentified	0.4	0.2

pentanol-formic acid with and without carrier citric acid. Further verification of the eluted compound as citrate was provided by coincidence of the acidic area (bromcresol green spray) with the radioactive area as shown by an autoradiogram. It is noted that Weinhouse *et al.* (42) isolated radioactive citrate (as the quinidine salt) from a variety of experimental neoplasms after incubation with isotopic glucose and palmitate.

The mouse hepatoma converted a large amount of the isotope of acetate-2-C^{14} to glutamate and aspartate and in this respect resembles the Gardner lymphosarcoma (20), but differed from the latter in that little if any of the isotope resided in glycine. Conversion of isotope to glycine might be demonstrated in hepatoma C_{984}, however, by adding glycine as carrier to the incubation medium. As pointed out by Kit (20), free glycine occurs in high concentration in lymphosarcomas,

and the large conversion of isotope to glycine in his experiments was no doubt facilitated by the "trapping" of isotopic glycine in this pool.

The heavy labeling of alanine and lactate, derivable from pyruvate by transamination and reduction, respectively, indicates that considerable isotope passed through the keto acid.

As much as 1.4 per cent of the nonvolatile residue resided in a ninhydrin-positive area which traveled close to the solvent front with phenol-water but which was immobile in the other direction. This is presumed to indicate the presence of a peptide, although this identification is tentative.

Utilization of Propionate-1-C^{14} by the Hepatoma

Two pathways have been proposed for the utilization of propionate by animal tissues: (*a*) oxidation to lactate via acrylate (16, 25) and (*b*) CO_2 fixation to yield succinate (22). 2-Methyl malonate (isosuccinate) has been shown to accumulate along with the succinate (14, 18). The present experiment was undertaken to see what radioactive compounds would arise from propionate-1-C^{14} incubated in the presence of slices of the hepatoma. The reproduction of the autoradiogram appears in Figure 5, and the data are given in Table 9. The nonvolatile fraction contained 1.5 per cent of the incubated isotope, while a much larger amount appeared in the respired CO_2, namely, 7.9 per cent.

TABLE 9

Utilization of Propionate-1-C^{14} by Hepatoma

(Conditions as in Table 3. Concentration of propionate-1-C^{14}, 0.0014 *M*; 1.4×10^7 counts/min incubated. 7.9 per cent of incubated counts/min recovered in CO_2; 1.5 per cent recovered in nonvolatile fraction.)

Per cent of nonvolatile fraction recovered as:*	
Lactate	43.0
Alanine	12.3
Glutamate	12.2
Citrate	8.2
β-Hydroxyvalerate	8.0
Aspartate	3.4
Origin	3.4
Glutamine	2.6
Urea?	1.5
Glucose	1.4
Unidentified	3.6

* Averaged from two chromatograms.

The most dominant radioactive compound on the chromatograms had a position identical with that of lactate. Over 40 per cent of the isotope of the nonvolatile residue was recovered in this compound. The next most active compound was alanine, with 13 per cent. The ratio of lactate-C^{14} to alanine-C^{14} was approximately equal to 3, a value close to that observed in experiments with acetate-1-C^{14} (Table 4). Acetate carbon can appear in lactate and alanine via pyruvate, and the fact that the ratio of lactate-C^{14} to alanine-C^{14} was the same, whether the two compounds were derived from propionate-1-C^{14} or acetate-1-C^{14}, suggests that propionate carbon passed through pyruvate before being converted to lactate. If this assumption is correct, α-oxidation of propionate to lactate (16, 25) is ruled out.

Eight per cent of the nonvolatile residue was recovered in an area close to that normally occupied by β-hydroxybutyrate. This is probably β-hydroxyvalerate, for Dans *et al.* (12) have shown that propionate-C^{14} is converted to this hydroxy acid by mouse liver slices, presumably from condensation of propionylSCoA with acetylSCoA followed by deacylation and reduction.

Oxidation of Uniformly Labeled Glucose by Slices of Hepatoma and Host Liver

The previous experiments were largely concerned with reactions closely associated with the tricarboxylic acid cycle. It was shown that the hepatoma cannot convert cycle intermediates, or even pyruvate, to glucose, at least not to any considerable extent. As it has long been observed that tumor tissues are active in glycolysis (38, 39), it became of interest to study the pattern of this glucose utilization by the chromatographic-autoradiographic procedure. Slices of hepatoma and host liver were incubated with a trace amount of uniformly labeled glucose, and the respiratory CO_2 and buffer extracts were analyzed. Typical autoradiograms for the hepatoma and host liver appear in Figures 6 and 7. The data are presented in Table 10.

Over 65 per cent of the isotope incubated with the hepatoma appeared as $C^{14}O_2$. This extraordinary ability of hepatoma to oxidize glucose was further substantiated upon inspection of the autoradiogram prepared from the chromatogram of the buffer extract. More isotope appeared in lactate and alanine than appeared in the residual glucose. On the other hand, the host liver converted only 13 per cent of the isotope to $C^{14}O_2$, indicating a much smaller utilization. In the case of host liver, very little isotope appeared in lactate or alanine, most of it residing in unreacted glucose. No exact measure was made of the per cent of residual glucose in either case. However, comparison of the two tissues with regard to isotope incorporation into lactate and alanine may be made by comparing the ratios of counts/min

in product:counts/min in residual glucose, as measured directly on the paper chromatograms (Table 10). The results indicate that hepatoma was much more active than host liver in the utilization of the *added* glucose.

The utilization of glucose by tumor in the present experiment exceeded that observed in a previous experiment (1). The greater utilization may be attributed to the fact that trace levels of glucose-C^{14} were employed here (less than 0.1 mg/flask), while in the previous experiments substrate levels were used.

In the case of hepatoma, isotope appeared also in glutamate. A group of radioactive spots occupied the region in which citrate is found. A radioactive area near the origin probably represents various phosphorylated derivatives.

A radioactive area just below glucose, termed "glucose artifact,"[1] was observed for both host liver and hepatoma. The intensity of this area seems to be a rather constant fraction of the radioactivity of the glucose area.

Whether or not all the isotopic carbon of $C^{14}O_2$ derived from the oxidation of glucose had traversed the tricarboxylic acid cycle in the case of tumor is not known. Recent experiments have indicated that this tumor may participate in reactions of the hexose monophosphate shunt (1).

The high capacity of the tumor to carry out glucose degradation may be responsible for the failure to obtain significant glucose synthesis from pyruvate and other compounds. Glycolytic intermediates derived from such compounds would have to traverse a metabolic gradient to reach the glucose level.

Glucose degradation via the Embden-Meyerhof scheme leads to pyruvate, which may enter the tricarboxylic acid cycle as oxaloacetate (44) or as malate (28). A rational explanation may thus be given for the high incorporation of isotope into the di- and tricarboxylic acids when tumor is incubated with a source of labeled 2-carbon fragments. The increase in pool size of cycle intermediates (*cf.* 45), resulting from increased glucose breakdown in tumor, could enable greater amounts of isotope to be trapped in the pools than is the case with normal or host liver. Aspartate and glutamate represent alternate pathways for oxaloacetate and α-keto-glutarate, respectively, and, in the tumor, an increased level of isotope in these amino acids is probably a reflection of the increase in their pool sizes.

[1] This was previously referred to as "glucose amine" (17) and has not yet been identified.

SUMMARY

1. The incorporation of isotope from acetate-1-C^{14}, acetate-2-C^{14}, propionate-1-C^{14}, octanoate-1-C^{14}, pyruvate-2-C^{14}, and from uniformly labeled glucose into respiratory CO_2 and various intermediary products was studied with slices of normal mouse liver, host liver, and with the hepatoma C_{954}.

2. A chromatographic-autoradiographic method was employed to demonstrate and identify about twelve or more labeled intermediates. Radioactive areas of the chromatograms, as indicated by autoradiograms, were assayed with a thin end-window Geiger tube.

3. In both liver and hepatoma, octanoate-1-C^{14} was utilized in preference to acetate-1-C^{14} as judged by (*a*) conversion to $C^{14}O_2$; (*b*) over-all incorporation into nonvolatile compounds; (*c*) incorporation into individual intermediary products; and (*d*) incorporation into acetoacetate.

4. Major C^{14} products (comprising more than 5 per cent of the nonvolatile residues) for the hepatoma incubated with octanoate-1-C^{14} were, in generally decreasing order of isotope incorporation, glutamate, di- and tricarboxylic acids (taken as a group), β-hydroxybutyrate, lactate, aspartate, and glutamine. Essentially the same pattern was found when acetate-1-C^{14} was the substrate. Quite striking was the value of the ratio of glutamate-C^{14} to glutamine-C^{14}, which was 6.7 for hepatoma, as compared with 0.6 for host liver. The hepatoma incorporated relatively larger amounts of isotope into di- and tricarboxylic acids,

TABLE 10

UTILIZATION OF UNIFORMLY LABELED GLUCOSE BY SLICES OF HEPATOMA AND HOST LIVER

(One-gm. slices incubated under conditions of Table 3. Glucose concentration, <0.1 mg. [trace]; 4.4 × 10^6 counts/min incubated. Tumors from several animals were pooled.)

Tissue	Respiratory CO_2: Counts/min	Respiratory CO_2: Per cent of incubated counts/min	Ratio of counts/min: Lactate/glucose	Ratio of counts/min: Alanine/glucose	Ratio of counts/min: Glutamate/glucose
Hepatoma	292,000	66	1.7*	1.5*	0.59*
Host liver	59,000	13	0.32†	0.027†	0†

* Average of three chromatograms.
† Average of two chromatograms.

glutamate, and aspartate than did the liver.

5. Normal and host liver incorporated isotope from labeled 2-carbon fragments into glucose, *but hepatoma failed to carry out this incorporation to any significant extent.* The hepatoma was more active than was host liver, however, in utilization of glucose.

6. Two-carbon fragments from acetate were shown to interact with those from octanoic acid in the hepatoma and to participate in the ketogenic activity of this short-chain fatty acid.

7. Alanine and lactate contained relatively more isotope when acetate-2-C^{14} was employed instead of acetate-1-C^{14}, with hepatoma. The explanation for this lies in the dynamics of the tricarboxylic acid cycle, the intermediates of which become more heavily labeled with the methyl carbon than with the carboxyl carbon of a 2-carbon fragment.

8. Propionate-2-C^{14} was converted by the hepatoma to (in decreasing order) lactate, alanine, glutamate, aspartate, glutamine, and several unidentified compounds. Little or no isotope was converted to glucose.

9. Presumptive evidence that the tricarboxylic acid cycle operates in the hepatoma was provided by (*a*) oxidation of labeled 2-carbon fragments to $C^{14}O_2$; (*b*) incorporation of isotope from 2-carbon fragments into compounds of the cycle and to those arising from them by ancillary processes; (*c*) inhibition by malonate; (*d*) depression in the oxidation of labeled acetate by short chain fatty acids which are thought to be inhibitors of the tricarboxylic acid cycle; and (*e*) the pattern of isotope labeling similar to that of liver, in which the cycle is known to operate.

10. The pattern of labeling of various intermediates observed when C^{14}-labeled compounds are incubated with slices of hepatoma differs quantitatively from the pattern observed with host or normal liver. The observations made here on quantitative differences in the hepatoma $C_{9.4}$ reflect its neoplastic state, but such differences from the liver tissue do not lend themselves to a clear-cut decision regarding the *specific loci* for the metabolic derangement(s) associated with neoplasia.

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Fig. 1.—Autoradiogram: Isotopic compounds derived from octanoate-1-C^{14} in presence of normal mouse liver slices.

Fig. 2.—Autoradiogram: Isotopic compounds derived from octanoate-1-C^{14} in presence of host liver slices.

Fig. 3.—Autoradiogram: Isotopic compounds derived from octanoate-1-C^{14} in presence of mouse hepatoma $C_{9.4}$ slices.

Fig. 4.—Autoradiogram: Isotopic compounds derived from acetate-2-C^{14} in presence of mouse hepatoma $C_{9.4}$ slices.

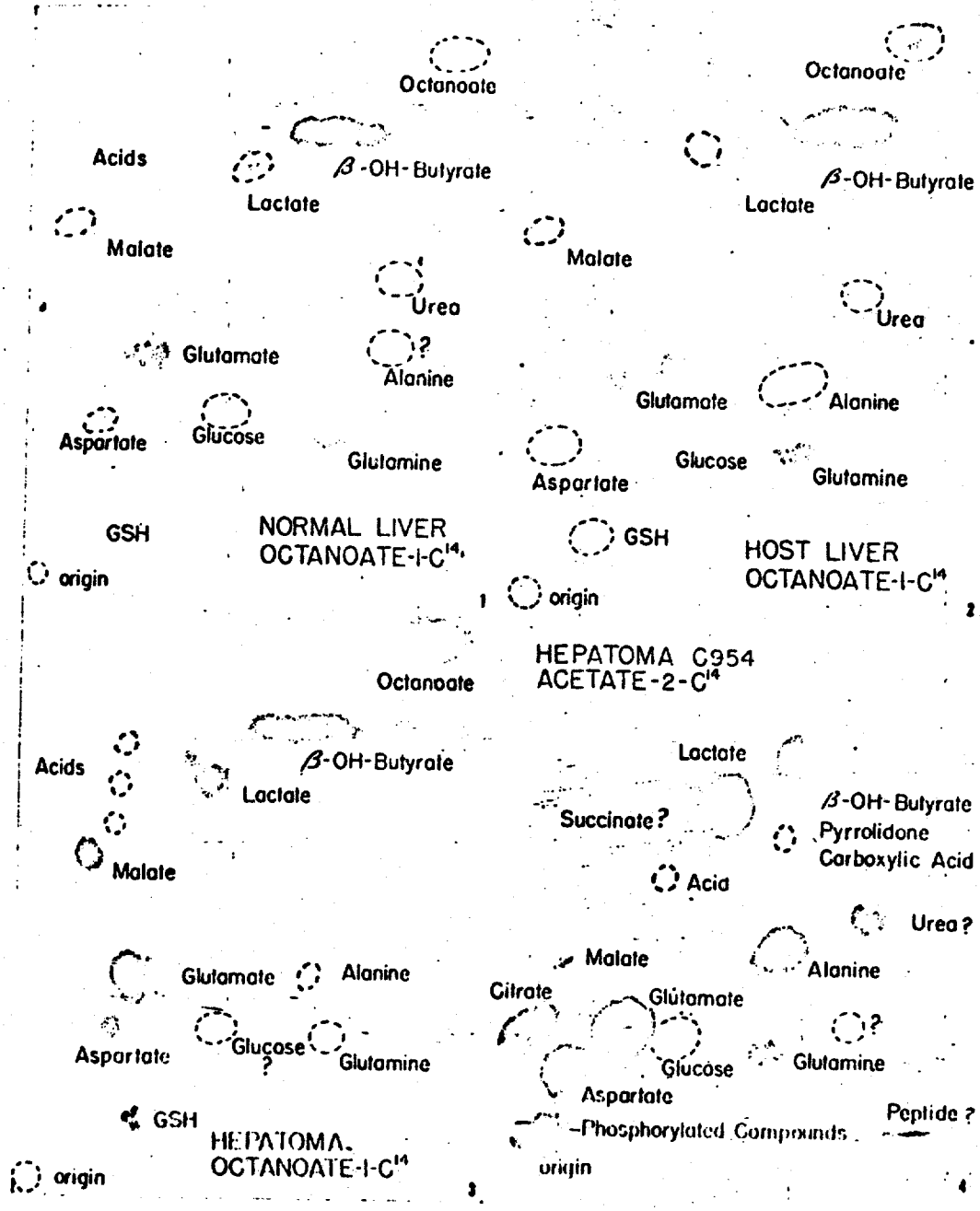
Octanoate
Acids
β-OH-Butyrate
Lactate
Malate
Urea
Glutamate
?
Alanine
Aspartate
Glucose
Glutamine
GSH
NORMAL LIVER
OCTANOATE-1-C14
origin
1
Octanoate
β-OH-Butyrate
Lactate
Malate
Urea
Glutamate
Alanine
Glucose
Glutamine
Aspartate
GSH
HOST LIVER
OCTANOATE-1-C14
origin
2
Octanoate
HEPATOMA C954
ACETATE-2-C14
Acids
β-OH-Butyrate
Lactate
Malate
Lactate
Succinate?
β-OH-Butyrate
Pyrrolidone
Carboxylic Acid
Acid
Urea?
Glutamate
Alanine
Aspartate
Glucose
?
Glutamine
GSH
HEPATOMA
OCTANOATE-1-C14
origin
3
Malate
Alanine
Citrate
Glutamate
?
Glucose
Glutamine
Aspartate
Peptide ?
-Phosphorylated Compounds
origin
4

FIG. 5.—Autoradiogram: Isotopic compounds derived from propionate-1-C^{14} in presence of mouse hepatoma C_{57} slices.

FIG. 6.—Autoradiogram: Isotopic compounds derived from evenly labeled glucose-C^{14} in presence of mouse liver slices.

FIG. 7.—Autoradiogram: Isotopic compounds derived from evenly labeled glucose-C^{14} in presence of mouse hepatoma C_{57} slices.

HEPATOMA C954
PROPIONATE-1-C^{14}

Succinate

Lactate

β-Oh-Valerate

HOST LIVER
GLUCOSE-E-C^{14}

Lactate

Alanine

Glutamate

Citrate ?

Glutamine

Unidentified Compounds

Glucose

Alanine

Glutamine

Glucose Artifact

Peptide ?

origin

origin

HEPATOMA C954
GLUCOSE-E-C^{14}

Lactate

Alanine

Citrate + ?

Glutamate

Glucose

Glucose Artifact

Phosphorylated Compounds

origin

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Effect of Free Fatty Acids and Insulin on Protein Synthesis and Amino Acid Metabolism of Isolated Rat Diaphragms

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SUMMARY

The effect of free fatty acids and insulin on the incorporation of C-14 from amino acids into muscle proteins was studied in vitro using "cut" or "intact" rat diaphragms.

Insulin promoted the incorporation of C-14 from L-glycine-2-C-14, L-histidine-2-ring-C-14 and L-leucine-U-C-14 into muscle proteins. Insulin stimulated the uptake of 0.05 mM. leucine-U-C-14 from the medium, but not its oxidation to C-14-O_2.

Palmitate (3 mM.) decreased the incorporation of C-14 from glycine and histidine into proteins of muscles stimulated with insulin. Octanoate (0.5-3 mM.) decreased the incorporation of C-14 from leucine into proteins. The inhibitory effect of octanoate on protein synthesis was demonstrated in the presence or absence of glucose and/or insulin. DL-β-hydroxybutyrate (15 mM.) decreased the incorporation of C-14 from leucine-U-C-14 into proteins in the presence of insulin.

Octanoate (1 mM.) stimulated the uptake of C-14 from leucine-U-C-14 from the medium and its oxidation to C-14-O_2. DL-β-hydroxybutyrate (15 mM.) appeared to inhibit both processes, however; the latter may be due to isotope dilution.

The nonutilizable amino acid, α-aminoisobutyric acid-C-14, penetrated less readily into the intracellular water of diaphragms incubated with 1 mM. octanoate than into controls. However, since octanoate stimulated the uptake of C-14 from leucine from the medium it appears likely that octanoate inhibited the incorporation of C-14 from leucine into proteins at a step beyond amino acid transport.

It is suggested that FFA can influence amino acid metabolism in muscles and decelerate their incorporation into proteins. Limitation of protein synthesis in muscle by increased intracellular FFA may contribute to the supply of amino acids available for neoglucogenesis during stress and carbohydrate deprivation. DIABETES *16*:753-64, November, 1967.

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Glucose utilization in muscle is regulated in part by the intracellular concentration of free fatty acids (FFA). Increased FFA oxidation inhibits glycolysis. This phenomenon has been described as the glucose-fatty acid cycle and has been implicated as a cause of insulin resistance during prolonged fasting, following treatment with corticosteroids and growth hormone and in uncontrolled diabetes mellitus.[1,2] With the exception of growth hormone treatment, the above conditions are accompanied by a negative nitrogen balance. The purpose of this study is to show that increased oxidation of FFA by muscle may influence amino acid metabolism in this tissue and may inhibit protein synthesis.

MATERIAL AND METHODS

Male rats of the Wistar strain, weighing 150 to 250 grams were used. In individual experiments the weight of the rats was as similar as possible, generally within 10 grams. The animals were fed ad libitum, (Wayne Lab blocks or Purina rat pellets) until they were sacrificed by a blow on the head. The diaphragms were removed immediately, rinsed free of blood with buffer, blotted and incubated. Three isolated diaphragm preparations were used. In the initial experiments on protein synthesis and in experiments concerning amino acid uptake and oxidation to CO_2 the two hemidiaphragms were dissected separately and incubated in separate vessels. This preparation will be referred to as the "cut" hemidiaphragm. In most of the experiments on protein synthesis and in studies of AIB transport the intact diaphragm preparation of Kipnis and Cori was used[3]; in most studies this preparation was divided along the midline.[4] The latter combines the advantage of minimizing the number of severed muscle fibers with the possibility of designing paired experiments. Thus, whenever "cut" or "intact hemidissected" diaphragms were used, one half was incubated with and the other without the fatty acid to be tested. Equal numbers of right and left half muscles were assigned to each medium.

The medium was Gey and Gey's balanced salt solution[5] (bicarbonate buffer) with the appropriate addi-

tions, equilibrated with O_2 and CO_2 (95:5), *pH* 7.4-7.5. Glucose when present in the medium was 1 mg. per milliliter and insulin* 1 or 100 mU. per milliliter. The incubation medium also contained a C-14 labeled amino acid (diluted as required with nonlabeled carrier), or other tracers such as sorbitol-H_3 and urea-C-14. Fatty acids (0.5-3 mM.) were added to alternate vessels. Octanoate and β-OH-butyrate were dissolved directly in the medium, following neutralization with equimolar NaOH. Palmitate was added to media containing crystalline bovine serum albumin (Armour) as a carrier. Palmitic acid was neutralized with a slight excess of NaOH, dissolved in a water bath at 60° C., and added dropwise to the medium with constant stirring.[6] In experiments involving palmitate, the control vessels (without added palmitate) also contained albumin. The FFA content of the albumin was measured by the method of Dole and Meinertz[7] and was 14.7 μmoles per gram.

The *pH* of the incubation media was measured before and at the end of the incubation period, by glass electrode, with the medium at 37.5° C., and at equilibrium with the gas phase. In some experiments the *pH* decreased slightly during incubation, generally less than 0.15 *pH*. There was no difference between the *pH* of control media and those containing FFA.

Following one-hour preincubation the tissues were gently blotted and transferred to vessels containing the incubation media. Generally, the preincubation media contained the same additions as the incubation media, except that the radioactive tracers and insulin were added to the latter only. Preincubation and incubation were in a Dubnoff metabolic shaker, at 37.5° C., with shaking (42 cycles per minute). Identical volumes were used for preincubation and incubation; these were 4 ml. medium for "cut" hemidiaphragms, 24 for with the intact diaphragm preparation and 12 ml. for its bisected modification. The purpose of preincubation was to alter the metabolic environment in the muscles (by increased fatty acid oxidation) before the amino acid tracer was added to the system.

Analytical procedures:

Tissue weight: When cut hemidiaphragms were used the tissues were weighed prior to incubation. The hemidiaphragms were placed into tared vessels containing the preincubation medium and reweighed to the nearest 0.1 mg. When the intact diaphragm or its bisected modification were used the hemidiaphragms were dissected free of the supporting tissues after incubation, rinsed for thirty seconds in buffer, blotted, placed into tared test tubes containing trichloroacetic acid (TCA), and weighed.

Incorporation of C-14 from amino acids into proteins: After two hours' incubation with a C-14 labeled amino acid the hemidiaphragms were placed into 5 ml. of 10 per cent TCA. Tissue proteins were isolated by TCA precipitation followed by extraction with hot TCA, performate digestion and reprecipitation with TCA.[8] In initial experiments the protein was suspended in a mixture of petroleum and ethyl ether (75:25) and placed on tared copper disks. The dried protein powder, aliquots of the medium and of the TCA supernatant were counted in a thin window gas flow counter. The planchets were reweighed and the counts corrected for self-absorption.

In later experiments, the dried protein powder was weighed and solubilized in Hyamine. One milliliter of Hyamine containing 3 to 5 mg. of protein, was added to 14 ml. of toluene scintillator* liquid and counted in a β-liquid scintillation counter. Aliquots of media before incubation (50λ) were also counted in the Hyamine-toluene-scintillator system. Counts were corrected for quenching with internal standards or by automatic external standardization. The radioactivity contained in the protein fraction was calculated as cpm per gram protein powder.

After incubation 0.2 ml. of each medium was added to 5 ml. of 10 per cent TCA. TCA extracts (0.5 ml.) of tissues and of media were added to 10 ml. of Bray's scintillation mixture[9] and counted. The nonprotein bound radioactivity in muscle was estimated by dividing the TCA soluble radioactivity contained per gram hemidiaphragm (wet weight) by the radioactivity contained per milliliter medium after incubation.

Studies concerning the transport of α-aminoisobutyric acid-C-14 (AIB) and the extracellular space: Following one-hour incubation with AIB-C-14, sorbitol-H-3 and urea-C-14 hemidiaphragms were dissected free of supporting tissues, weighed and extracted with 2 ml. of boiling 5 per cent TCA for five minutes. TCA extracts of tissues and media after incubation were counted in Bray's scintillation mixture. The space of distribution of AIB, sorbitol or urea was expressed as the fraction of the wet weight of the tissue which would be at equilibrium with the medium at the end of incubation.

*Five times recrystallized beef insulin (25.4 U./mg.) was generously supplied by Dr. W. R. Kirtley, Lilly Research Laboratories, Indianapolis, Indiana.

*Five grams of 2,5-Diphenyloxazole (PPO) and 0.3 gm. of 1,4-bis-2 (4-Methyl-5-Phenyloxazolyl) benzene (dimethyl POPOP) dissolved in one liter of toluene.

$$Space = \frac{cpm/gm.\ tissue}{cpm/ml.\ medium} \times 100.$$

AIB-C-14 and sorbitol-H-3 spaces were measured simultaneously on the same tissue sample, by differential counting of the two isotopes in a three channel instrument. Quench corrections were made by external standardization.

Tissue water was determined as the space of distribution of urea-C-14 or as the difference between the wet weight and the dry weight of the tissues following incubation. Dry weight was obtained after drying in an oven at 110° C. for two hours. Tissue water was measured under identical conditions as the AIB spaces but in separate experiments.

Uptake of C-14 from leucine-C-14 and conversion to C-14-O_2: "Cut" hemidiaphragms were incubated in medicinal flasks sealed with rubber stoppers equipped with sleeves. Small plastic cups were attached with stainless steel wire to the inside of the stoppers. The flasks were gassed through a needle with O_2 and CO_2 (95:5) for five minutes at the beginning of incubation and sealed. After two hours 0.5 ml. of Hyamine was injected into the plastic cups and 0.5 ml. 10 N H_2SO_4 into the medium. The flasks were incubated for an additional hour at 37.5° C. The plastic cups containing the C-14-O_2 trapped in Hyamine were placed in counting vials containing 14 ml. of toluene scintillator mixture and counted. Aliquots of the incubation medium, obtained before and after incubation were also counted in the Hyamine-toluene system. C-14 uptake was calculated as the cpm that disappeared from the medium during incubation. Uptake and oxidation to CO_2 were related to the initial wet weight of the tissue.

Reagents: C-14 and H-3 labeled chemicals were purchased from the New England Nuclear Corporation and the Nuclear Chicago Corporation. Palmitic and octanoic acids (Grade A) were purchased from the Calbiochem Corporation; DL-β-hydroxybutyric acid (sodium salt) from the Sigma Corporation and Mann Research Laboratories. Fatty acids were stored at 4° C. protected from light and humidity. Octanoate was stored under a nitrogen atmosphere. Analytical reagents were used in all preparations.

Presentation of data: Duplicate samples were prepared and counted in all studies.

Means ± the standard errors of the means are tabulated. The *t* test of significance was carried out according to Fisher and Yates. The number of tissues used for observation is indicated in parenthesis.

RESULTS

The effect of palmitate, albumin and insulin on protein synthesis (table 1)

Insulin stimulated the incorporation of C-14 from glycine and histidine into proteins of rat diaphragms ($p < 0.01$). This confirms studies of others. (For review of literature see references 10 & 11). Insulin increased the accumulation of nonprotein bound (TCA soluble) C-14 from glycine in the tissues ($p < 0.01$), but did not affect the accumulation of C-14 from histidine.[12]

Bovine serum albumin (2.5 gm. per cent) decreased the incorporation of C-14 from glycine into proteins and decreased the accumulation of TCA soluble C-14 from glycine in the tissues. These effects of albumin were significant ($p < 0.01$) only when protein synthesis was stimulated by the addition of insulin to the medium.

Albumin did not affect the incorporation of C-14 from histidine into proteins. However, the accumulation of TCA soluble C-14 from histidine in the tissues was significantly decreased by albumin in the presence or absence of insulin ($p < 0.01$). Albumin (1 gm. per cent) added to a medium containing insulin and leucine-U-C-14 did not affect the incorporation of C-14 into proteins, nor the accumulation of TCA soluble C-14 in the tissues. Inhibition of the incorporation of C-14 from glycine into proteins by 5 gm. per cent bovine serum albumin has been reported, and attributed to nonspecific amino acid competition.[13]

The addition of 3 mM. palmitate to a medium containing albumin decreased the incorporation of C-14 from glycine or histidine into proteins. This effect was not significant when the incubation was carried out without insulin in the medium. However, when 3 mM. palmitate was added to a medium containing insulin and albumin, the hemidiaphragms incorporated 10 to 20 per cent less C-14 glycine or histidine into proteins than controls incubated with albumin and insulin ($p < 0.05$).

Palmitate (1 mM.) added to a medium containing insulin and albumin had no significant effect on the incorporation of C-14 from leucine-U-C-14 into proteins. The concentration of nonprotein bound C-14 from leucine-U-C-14 was slightly but significantly greater ($p < 0.01$) in diaphragms incubated with 1 mM. palmitate, albumin and insulin than in controls incubated with albumin and insulin.

The effect of octanoate and insulin on protein synthesis (table 2)

TABLE 1

The effect of palmitate on the incorporation of C-14 from amino acids into proteins of isolated rat diaphragms

Experiment	Diaphragm preparation	Amino acid μM.		Additions to medium* Albumin mg/ml.	FFA mM.	Insulin mU./ml.	C-14 incorporation into proteins mμg./gm. protein†	FFA effect p‡	Nonprotein bound C-14 in muscle "Space" ± S.E.M.§
A	"Cut"	L-glycine-2-C-14 2 μM.	a	—	—	—	5.94 ± 0.46 (6)		240 ± 11.1 (6)
			b	25	—	—	5.22 ± 0.32 (6)	c < b	218 ± 7.1 (6)
			c	25	3	—	4.88 ± 0.16 (6)	n.s.	207 ± 7.0 (6)
			d	—	—	100	10.34 ± 0.16 (6)		370 ± 22.0 (6)
			e	25	—	100	9.18 ± 0.24 (6)	f < e	282 ± 11.1 (6)
			f	25	3	100	8.26 ± 0.28 (6)	< 0.05	287 ± 15.9 (6)
B	"Cut"	L-histidine 2-ring-C-14 140 μM.	a	—	—	—	424.0 ± 30.8 (8)		421 ± 38.7 (8)
			b	25	—	—	416.0 ± 14.0 (8)	c < b	334 ± 11.8 (8)
			c	25	3	—	389.0 ± 30.8 (8)	n.s.	290 ± 19.6 (8)
			d	—	—	100	529.0 ± 29.4 (8)		450 ± 21.5 (8)
			e	25	—	100	519.0 ± 21.0 (8)	f < e	340 ± 23.8 (8)
			f	25	3	100	434.0 ± 18.0 (8)	< 0.05	323 ± 30.9 (8)
C	"Intact bisected"	L-leucine-U-C-14 50 μM.	d	—	—	1	583.0 ± 48.5 (8)		91 ± 3.5 (12)
			e	12.5	—	1	616.0 ± 33.5 (11)	f < e	86 ± 2.3 (18)
			f	12.5	1	1	589.0 ± 26.5 (11)	n.s.	97 ± 4.4 (18)

*Diaphragms were preincubated (1 hr.) and incubated (2 hrs.) in balanced salt solution[5] containing glucose (1 mg./ml.). Albumin and palmitate were added during both phases; insulin and amino acids only during the incubation period. The amino acid concentrations indicated include the labeled amino acid (0.05-0.2 μc./ml.) and the nonlabeled carrier.
†cpm./gm. tissue protein × amino acid mμmoles/cpm in medium before incubation.
‡p values for insulin effect on C-14 incorporation into proteins. In study A: d > a, e > b, f > c = each p < 0.01; (e-b) > (f-c) = n.s. In study B: d > a, e > b = each p < 0.01. f > c = n.s. (e-b) > (f-c) = n.s. Albumin effect: only significant difference e < d (p < 0.01) in study A.
§TCA sol. cpm/gm. tissue / cpm/ml. medium after incubation.
Significant p values in "spaces": In study A: d > a, e > b, f > c (each p < 0.01, insulin effect). e < d (p < 0.01, albumin effect). In B: b < a, e < d, (each p < 0.05, albumin effect). In C: f > e (p < 0.01, FFA effect).

As reported previously[10,11,14] the incorporation of C-14 from leucine into proteins was significantly stimulated by the addition of 1 mU. of insulin to the medium ($p < 0.01$).

The addition of octanoate to an insulin containing medium significantly decreased the incorporation of C-14 from leucine into proteins ($p < 0.01$ in studies A-G). This was demonstrated with "cut" hemidiaphragms and 3 mM. octanoate (study A) and with the "intact diaphragm" preparation and 0.5 or 1 mM. octanoate (studies B-G). It occurred in the presence of glucose in the medium (studies A, B, D-G) and in its absence (study C). Although insulin significantly stimulated protein synthesis in the presence of octanoate in study A ($p < 0.05$), paired analysis of the data showed that the effectiveness of insulin was diminished by octanoate ($p < 0.05$).

In the absence of insulin, octanoate decreased the C-14 incorporated into proteins slightly. This effect was not significant when the medium contained glucose in addition to FFA (studies A & B). However, in study C, which was carried out without glucose in the medium, 1 mM. octanoate significantly inhibited the incorporation of C-14 from leucine into proteins even in the absence of insulin ($p < 0.01$).

The concentration of leucine in the medium was 0.05 mM. in each study in table 2, except F and G. (The leucine content of rat serum has been reported as 0.16 mM.)[11]

Leucine was increased to 0.5 mM. in study F to ascertain whether or not the effect of octanoate on C-14 incorporation into proteins could represent isotopic dilution, instead of inhibition of protein synthesis. During incubation with 1 mM. octanoate-1-C-14 approximately 0.7 per cent of the C-14 which was taken up by the diaphragms was incorporated into protein (table 3). This represented 0.1 μmole of octanoate per gram tissue in two hours or 0.5 μmole per gram protein. Fragments of octanoate molecules may have entered the leucine pool by transamination. During incubation with 0.05 mM. leucine-U-C-14, approximately 50 per cent of the radioactivity that disappeared from the medium was incorporated into proteins (compare table 5 and table 2). This represented 0.5 to 1.2 μmoles of leucine per gram

TABLE 2

The effect of octanoate on the incorporation of C-14 from leucine into proteins

Experiment	Diaphragm preparation	Control medium* Leucine μM. (label)	Glucose mg./ml.	Additions	C-14 incorporations into proteins: mμmoles/gm. protein† Mean ± S.E.M.	FFA effect d ± S.E.M.	p‡
A	"Cut"	50 (U-C-14)	1	a Control	800± 89 (7)		
				b + 3 mM. FFA	705± 39 (7)	— 95	n.s.
				c + insulin	1,205±131 (6)		
				d + FFA + insulin	850± 51 (7)	—355± 92	<0.01
B	"Intact"	50 (U-C-14)	1	a Control	530± 41 (8)		
				b + 1 mM. FFA	460± 67 (8)	— 70	n.s.
				c + insulin	870±107 (8)		
				d + FFA + insulin	635±117 (8)	—235± 61	<0.01
C	"Intact bisected"	50 (U-C-14)	—	a Control	585± 20 (12)		
				b + 1 mM. FFA	495± 30 (12)	— 90± 22	<0.01
				c + insulin	765± 79 (12)		
				d + FFA + insulin	595±163 (12)	—170± 40	<0.01
D	"Intact bisected"	50 (U-C-14)	1	c Control + insulin	1,045± 70 (14)		
				d + 0.5 mM. FFA + ins.	865± 66 (14)	—180± 34	<0.01
E	"Intact bisected"	50 (1-C-14)	1	c Control + insulin	525± 41 (8)		
				d + 1 mM. FFA + ins.	400± 28 (8)	—125± 58	<0.01
F	"Intact bisected"	500 (U-C-14)	1	c Control + insulin	3,250±130 (10)		
				d + 1 mM. FFA + ins.	2,350± 75 (10)	—900±130	<0.01
G	"Intact bisected"	500 (U-C-14) No preincubation	1	c Control + insulin	1,950±120 (10)		
				d + 1 mM. FFA + ins.	1,550±110 (10)	—400± 95	<0.01

*Diaphragms were preincubated (1 hr.) and incubated (2 hrs.) in balanced salt solution[5] with the indicated additions. Octanoate and glucose were added during both phases, leucine-C-14, carrier leucine and insulin (1 mU./ml. when present) during incubation only. Leucine-C-14 = 0.05 μc./ml. = 0.25 μM. (Spec. act. > 200 mc./mmole). The concentrations of carrier leucine are indicated.

†cpm/gm. tissue protein × leucine mμmoles/cpm in medium before incubation.

‡p = paired analysis. p values for insulin effects: in study A: c > a = p < 0.01; d > b = p < 0.05; (c-a) > (d-b) = p < 0.05. In study B: c > a = p < 0.01, d > b = n.s.; (c-a) > (d-b) = n.s. In study C the effect of octanoate in the presence or absence of insulin was studied in separate experiments.

protein in two hours. The inhibition of protein synthesis observed in the presence of 1 mM. octanoate was in the order of 15 to 30 per cent. Therefore, an isotope dilution effect could not be ruled out completely.

Study F in table 2 disproves this hypothesis. The concentration of leucine was 0.5 mM. and 3.2 μmoles of leucine were incorporated per gram protein in two hours. The addition of 1 mM. octanoate to the medium decreased the incorporation of C-14 from leucine into proteins by 0.9 μmoles per gram protein. A tenfold increase in the leucine concentration of the medium did not diminish the inhibitory effect of FFA on the incorporation of C-14 from leucine into proteins suggesting that the octanoate induced inhibition is exerted on protein synthesis per se.

In every study except G in table 2 the two-hour incubation period in the presence of the radioactive tracer was preceded by one-hour preincubation with all the ingredients to be tested except the C-14 labeled amino acid, its nonlabeled carrier and insulin. Study G was carried out under identical conditions as study F except that the period of preincubation was omitted. The diaphragms which had not been preincubated incorporated less C-14 from leucine into proteins (study G) than the diaphragms which had been preincubated for one hour without the radioactive tracer (study F). The two studies were carried out several months apart and this difference may be due to a noncontrolled variable. However, it is possible that the endogenous stores of leucine decreased during the one-hour preincubation resulting in heavier labeling of the newly formed protein during the subsequent incubation period. Although this point is not resolved, study G shows clearly that preincubation is not a prerequisite for the inhibitory effect of octanoate on protein synthesis. Diaphragms which were not preincubated incorporated 23 per cent less C-14 into proteins in the presence of octanoate than the controls ($p < 0.01$), while octanoate decreased C-14 incorporation by 28 per cent in the preincubated series ($p < 0.01$).

TABLE 3

Metabolism of C-14 from octanoate-1-C-14 by diaphragms

		μmoles/gm. tissue/2 hrs.
(A)	C-14 uptake from medium	14.01±0.44 (8)
(B)	Oxidation to C-14-O_2	4.29±0.17 (8)
(C)	Incorporation into protein	0.10±0.004 (4)

The medium was balanced salt solution[5] containing 1 mM. nonlabeled octanoate during preincubation (1 hr.); octanoate-1-C-14 (0.25 μc./ml.) with nonlabeled octanoate (final conc. 1 mM.) and 5 × 10^{-2} mM. nonlabeled leucine during incubation (2 hrs.). A and B were determined simultaneously, using "cut" hemidiaphragms and 4 ml. of medium. "Intact hemidissected" diaphragms were incubated in 12 ml. of medium in C. Results in C were originally determined per milligram protein. The calculation assumes that protein constitutes 18 per cent of the weight of the tissue.

The effect of DL-β-hydroxybutyrate on protein synthesis (table 4)

DL-β-hydroxybutyrate was less effective than octanoate in inhibiting protein synthesis. In preliminary experiments (not shown in table 4), 1 or 4 mM. DL-β-hydroxybutyrate did not affect the incorporation of C-14 from leucine into proteins. DL-β-hydroxybutyrate decreased the incorporation of C-14 from leucine into proteins when added at a 15 mM. concentration to media containing insulin. The inhibition was of borderline significance ($0.05 < p < 0.1$) when glucose was also present in the medium, but was significant in the absence of glucose ($p < 0.01$). In the absence of insulin, β-hydroxybutyrate did not affect the incorporation of C-14 into proteins.

The effect of octanoate, β-hydroxybutyrate and insulin on leucine uptake and oxidation

C-14 uptake and oxidation were studied during incubation with 0.05 mM. leucine-U-C-14 (table 5). Insulin stimulated the disappearance of C-14 from leucine-U-C-14 from the medium ($p < 0.01$).[14] Insulin did not affect the oxidation of leucine-U-C-14 since the cpm recovered in C-14-O_2 were essentially identical during incubation with and without insulin. Presumably the extra leucine-C-14 that was taken up by the diaphragm under the influence of insulin was incorporated into proteins and not made available for oxidation.

Ocanoate 1 mM., added to the medium in the absence of insulin, caused the diaphragms to utilize 40 per cent more C-14 from the medium than the controls ($p < 0.01$) and increased the oxidation of C-14 from leucine by 200 per cent ($p < 0.01$). While in control muscles 15 to 20 per cent of the C-14 that disappeared from the medium was recovered as C-14-O_2, in the presence of octanoate the oxidized fraction was doubled. Although octanoate appeared to enhance the uptake of C-14 from leucine more than insulin, this difference was not significant. Octanoate added to an insulin containing medium caused no significant further stimulation of leucine uptake. However, octanoate in the presence of insulin increased the fraction of C-14 from leucine that was oxidized by 160 per cent ($p < 0.01$).

The effect of β-hydroxybutyrate was the opposite of that of octanoate. β-hydroxybutyrate decreased the uptake of C-14 from leucine-U-C-14 by 18 to 19 per cent in the absence and in the presence of insulin ($p < 0.01$). It

TABLE 4

The effect of DL-β-hydroxybutyrate on the incorporation of C-14 from leucine-U-C-14 into proteins

Experiment		Additions to medium* Glucose 1 mg./ml.	DL-β-OH-butyrate 15 mM.	Insulin 1 mU./ml.	C-14 incorporation into proteins mμmoles/gm. protein† Mean ± S.E.M.	FFA effect d ± S.E.M.	p‡
A	a	+	—	—	615± 30 (8)		
	b	+	+	—	670± 51 (8)	+ 55	n.s.
	c	+	—	+	945± 80 (7)		
	d	+	+	+	840±106 (7)	—105±49	<0.1
B	a	—	—	—	505± 55 (14)		
	b	—	+	—	555± 74 (14)	+ 50	n.s.
	c	—	—	+	680± 33 (12)		
	d	—	+	+	555± 47 (12)	—125±36	<0.01

"*Intact bisected" diaphragm preparations were preincubated (1 hr.) and incubated (2 hrs.) in balanced salt solution[5] with the indicated additions. Insulin, leucine and leucine-U-C-14 were added only during the incubation period. Leucine-U-C-14 = 0.05 μc./ml. = 0.25 μM. Nonlabeled carrier leucine = 50 μM.

†cpm/gm. tissue protein × leucine mμmoles/cpm in medium before incubation.

‡p values for insulin effect. In Study A: c > a = p < 0.01, d > b = n.s. In Study B: c > a = p < 0.02, d = b (n.s.)

also caused a 50 per cent decrease in the oxidation of C-14 from leucine-U-C-14 ($p < 0.01$).

The effect of octanoate, β-hydroxybutyrate and insulin on the accumulation of TCA-soluble C-14 from leucine in rat diaphragms (table 6)

In each experiment where the tracer used was 0.05 mM. uniformly labeled leucine-C-14 the addition of octanoate to the medium promoted the accumulation of TCA soluble radioactivity in the tissues ($p < 0.01$, studies A-D). This occurred in the presence or in the absence of insulin; with "cut" or "intact" diaphragms and with concentrations of octanoate varying from 0.5 to 3 mM. When the concentration of leucine in the medium was increased to 0.5 mM., the addition of octanoate did not affect the accumulation of TCA soluble C-14 from leucine-U-C-14 in the tissues (study E).

While octanoate enhanced the accumulation of TCA soluble C-14 from leucine-U-C-14 in the tissues, β-hydroxybutyrate decreased it. In each study where the tracer was 0.05 mM. leucine-U-C-14, less TCA soluble radioactivity accumulated in the tissues incubated with β-hydroxybutyrate than in the controls. This occurred in the presence or absence of insulin and/or glucose in the medium (studies F and G, $p < 0.01$).

Manchester has shown that although rat diaphragms oxidize a considerable portion of the leucine skeleton, C-1 is oxidized preferentially.[15] It appeared probable that the difference in the TCA soluble C-14 content of hemidiaphragms incubated with octanoate or β-hydroxybutyrate reflected a difference in the concentration of C-14 labeled fragments of the leucine-U-C-14 molecule which accumulated during leucine oxidation.

To test this hypothesis hemidiaphragms were incubated with octanoate or β-hydroxybutyrate and 0.5 mM. leucine-1-C-14 instead of leucine-U-C-14 as a tracer (study H). Under these conditions the effects of octanoate and β-hydroxybutyrate on the accumulation of TCA soluble C-14 were reversed. Octanoate significantly decreased the accumulation of TCA soluble C-14 from leucine-1-C-14 ($p < 0.01$) while β-hydroxybutyrate increased it ($p < 0.01$). This suggests that the concentration of TCA soluble C-14 from leucine-1-C-14 in the muscles was inversely proportional to the rate of oxidation of leucine.

During incubation with leucine-1-C-14 and insulin but without FFA the space of distribution of nonprotein bound C-14 in the tissues was 33 per cent less than the "spaces" obtained with leucine-U-C-14 under identical experimental conditions. The difference presumably represents the accumulation of C-14 labeled fragments of leucine-U-C-14 in the tissue water. Akedo and Christensen incubated diaphragms with 0.5 mM. leucine-C-14 and reported that more than 90 per cent of the nonprotein bound radioactivity in the tissues belonged to the unaltered amino acid.[12] They did not specify the position of the label on the leucine molecule. The apparent space

TABLE 5

The effect of fatty acids on the uptake and oxidation of C-14 from leucine-U-C-14 by isolated rat diaphragms

		FFA	mM.	Insulin mU./ml.	C-14 uptake* mμmoles/gm. tissue	FFA effect p‡	C-14-O_2 production mμmoles/gm. tissue†	FFA effect p‡	C-14-O_2 × 100 / C-14 uptake
A	a	Octanoate	—	—	210± 7 (8)		41±0.2 (8)		19.5
	b		1	—	291±11 (8)	↑p<0.01	123±4.7 (8)	↑p<0.01	42.3
	c		—	1	263±16 (8)		35±2.1 (8)		13.3
	d		1	1	296±17 (8)	↑n.s.	103±7.9 (8)	↑p<0.01	34.8
B	a	DL-β-OH-	—	—	228± 6 (4)		34±4.0 (4)		15.0
	b	butyrate	15	—	188± 5 (4)	↓p<0.01	15±0.2 (4)	↓p<0.01	8.1
	c		—	1	340±20 (8)		33±2.1 (8)		9.7
	d		15	1	274±20 (8)	↓p<0.05	16±0.1 (8)	↓p<0.01	5.9

Hemidiaphragms were incubated for two hours in 4 ml. of balanced salt solution containing glucose 1 mg./ml., L-leucine-U-C-14 (50 mμmoles/ml. = 0.1 μc./ml.), and the additions indicated in the table. Preincubation (1 hr.) was in identical media, but without leucine-C-14 or insulin.

*C-14 uptake = 4 (cpm/ml. IM—cpm/ml. FM) /gm. tissue/2 hrs. × leucine mμmoles/cpm IM, where IM is the initial medium and FM the medium after 2 hrs. incubation.

†C-14-O_2 production = cpm/gm. tissue/2 hrs. × leucine mμmoles/cpm in medium before incubation.

‡p values for insulin effect. In C-14 uptake, Study A: c > a = p < 0.01, d > b = n.s. In Study B: c > a = p < 0.01, d > b = p < 0.01. In C-14-O_2 production = no significant insulin effect.

TABLE 6

The effect of octanoate and DL-β-hydroxybutyrate on the accumulation of nonprotein bound C-14 from leucine in isolated rat diaphragms

Experiment	Control medium *Leucine μM. (label)	Glucose mg./ml.	Additions	Nonprotein bound C-14 in muscle "Space" ± S.E.M.†	FFA effect p‡
A	50 (U-C-14)	1	a Control	119± 6.2 (7)	
			b + 3 mM. octanoate	151± 4.9 (7)	↑p<0.01
			c + insulin	94± 6.4 (7)	
			d + octanoate + insulin	150± 8.4 (7)	↑p<0.01
B	50 (U-C-14)	1	a Control	114± 5.7 (8)	
			b + 1 mM. octanoate	141±11.2 (8)	↑p<0.05
			c + insulin	84± 2.3 (8)	
			d + octanoate + insulin	120±10.0 (8)	↑p<0.01
C	50 (U-C-14)	—	a Control	100± 3.2 (12)	
			b + 1 mM. octanoate	140± 3.8 (12)	↑p<0.01
			c + insulin	91± 2.8 (10)	
			d + octanoate + insulin	137± 3.3 (10)	↑p<0.01
D	50 (U-C-14)	1	c Control + insulin	87± 1.9 (15)	
			d + 0.5 mM. octanoate + insulin	128± 3.7 (15)	↑p<0.01
E	500 (U-C-14)	1	c Control + insulin	131± 3.5 (10)	
			d + 1 mM. octanoate + insulin	127± 5.7 (10)	n.s.
F	50 (U-C-14)	1	a Control	117± 5.7 (8)	
			b + 15 mM. DL-β-OH-butyrate	80± 1.6 (8)	↓p<0.01
			c + insulin	89± 3.3 (8)	
			d + β-OH-butyrate + insulin	75± 1.8 (8)	↓p<0.01
G	50 (U-C-14)	—	a Control	122± 3.6 (14)	
			b + 15 mM. DL-β-OH-butyrate	99± 3.1 (14)	↓p<0.01
			c + insulin	95± 2.0 (12)	
			d + β-OH-butyrate + insulin	79± 1.6 (12)	↓p<0.01
H	50 (1-C-14)	1	c Control + insulin	60± 1.4 (8)	
			d + 1 mM. octanoate + insulin	51± 1.4 (8)	↓p<0.01
			e + 15 mM. DL-β-OH-butyrate + insulin	73± 1.2 (8)	↑p<0.01

*L-leucine-U-C-14 or 1-C-14 = 0.05 μc/0.25 μM. The concentrations of carrier leucine are shown. The tissues were preincubated for one hour and incubated for two hours in balanced salt solution with the indicated additions. Leucine-C-14, carrier leucine and insulin (1 mU./ml. when present) were added only during incubation. "Cut" hemidiaphragms were used in study A, "intact diaphragms" in B, and "intact bisected" preparations in C-H.

$$\text{†Space} = \frac{\text{TCA sol. cpm/gm. tissue}}{\text{cpm/ml. medium after incubation}} \times 100.$$

‡p values for insulin effects. In studies A, B, C, F, and G: c < a = (in each $p < 0.01$). d < b = n.s. in studies A, B, C and F; $p < 0.01$ in G.

of distribution reported by these authors is similar to that observed in study H using 0.05 mM. leucine-1-C-14, and much lower than that observed using 0.5 mM. leucine-U-C-14 in study E.

Insulin without added FFA significantly decreased the accumulation of TCA soluble C-14 in diaphragms incubated with 0.05 mM. leucine-U-C-14 ($p < 0.01$ in studies A, B, C, F & G). In the presence of octanoate, insulin did not affect the accumulation of C-14 from leucine-U-C-14 in the diaphragm (Studies A-C).

The effect of octanoate and β-hydroxybutyrate on AIB transport

Since diaphragms incubated with octanoate took up more leucine-C-14 from the medium than the controls, the inhibitory effect of octanoate on the incorporation of C-14 from leucine into proteins cannot be ascribed to

decreased penetration of the amino acids into muscle cells. Indeed, it is necessary to postulate that octanoate influenced amino acid metabolism primarily beyond the transport step into muscle cells. The effect of octanoate and β-hydroxybutyrate on the penetration of a nonutilizable amino acid, α-aminoisobutyric acid-3-C-14 (AIB) into muscle cells is shown in table 7. The transport of AIB is known to be accelerated by insulin.[12,16]

Diaphragms incubated with 3 mM. octanoate showed a small but significant increase in the space of distribution of sorbitol and a slight increase in total tissue water. This suggests that high concentration of octanoate interfered with the integrity of the cell membrane. In addition 3 mM. octanoate inhibited the stimulatory effect of insulin on the transport of AIB ($p < 0.01$).

Incubation with 1 mM. octanoate did not affect the space of distribution of sorbitol or the water content of the diaphragms. Diaphragms incubated with 1 mM. octanoate responded to insulin with stimulation of AIB penetration, which is further proof of the relative integrity of the cell membrane. Octanoate (1 mM.) decreased the penetration of AIB into the tissue water of muscle cells by approximately 20 per cent in diaphragms incubated with or without insulin ($p < 0.01$, paired analysis).

DL-β-hydroxybutyrate (15 mM.) had no effect on the space of distribution of sorbitol or on the water content of the diaphragms. However, β-hydroxybutyrate increased the concentration of C-14 from AIB in muscle cells significantly, in the absence of insulin and when the transport of AIB was stimulated by insulin ($p < 0.01$, paired analysis).

C-14-O_2 production from AIB-C-14 (or from contaminants of the preparation) was measured in one experiment. "Cut" hemidiaphragms incubated without insulin or FFA oxidized 4.3 per cent of the C-14 contained per milliliter medium per gram tissue per hour when the AIB concentration was 8.5×10^{-3} mM. (Under the same conditions hemidiaphragm oxidized 82 per cent of the radioactivity contained per milliliter medium per gram tissue per two hours when the tracer was 5×10^{-2} mM. leucine-U-C-14). The addition of octanoate (1 mM.) or β-hydroxybutyrate (15 mM.) decreased the oxidation of AIB-3-C-14 (or its labeled contaminant) from 0.36 mμmoles per gram tissue per hour to 0.21 mμmoles per gram tissue per hour. The effects of the two FFA on oxidation were essentially the same. Since 1 mM. octanoate decreased the concentration of C-14 from AIB in the intracellular water, and reduced the small oxidized fraction of AIB-C-14, it is probable

TABLE 7

The effect of octanoate and β-hydroxybutyrate on the penetration of AIB into rat diaphragms

	FFA	μmoles/ml.	Glucose mg./ml.	Insulin mU./ml.	AIB space* Per cent of wet wt. ± S.E.M.	Sorbitol space* Per cent of wet wt. ± S.E.M.	Tissue water* Per cent of wet wt. ± S.E.M.	"Intracellular AIB space"†
A	Octanoate	—	1	—	73.3±3.5 (8)	27.3±0.7 (8)	78.5±0.2 (8)	89.7
		3	1	—	81.3±3.3 (8)	32.6±1.2 (8)	80.7±1.0 (8)	101.3
		—	1	1	124.2±3.9 (8)	28.0±1.3 (8)	77.6±0.4 (8)	193.9
		3	1	1	93.0±7.4 (8)	33.6±2.3 (8)	79.2±0.4 (8)	130.2
B	Octanoate	—	1	—	57.7±2.6 (12)	24.4±3.1 (12)	75.2±1.1 (10)	65.6
		1	1	—	51.0±2.0 (12)	24.7±1.1 (12)	76.2±1.0 (10)	50.9
		—	1	1	85.0±5.7 (12)	23.3±0.6 (12)		119.0
		1	1	1	73.4±4.2 (12)	25.8±1.5 (12)		94.5
C	DL-β-hydroxybutyrate	—	—	—	53.6±2.4 (8)	24.2±1.2 (8)	75.2±1.1 (10)	57.8
		15	—	—	62.7±3.5 (8)	23.3±1.6 (8)	74.0±1.2 (10)	77.7
		—	—	1	90.1±5.2 (8)	26.1±1.8 (8)		140.3
		15	—	1	111.3±8.1 (8)	26.0±2.5 (8)		177.8

Intact diaphragms were used in study A. and "Intact hemidissected" in B and C. AIB = α-aminoisobutyric acid-3 C-14, 8.5×10^{-3} mM. (0.04 μc./ml.). Sorbitol-H^3 = 6×10^{-3} mM. (0.5 μc./ml.). The Sorbitol and AIB spaces were measured simultaneously in studies B and C, but not in A. Tissue water was determined in separate experiments; as wet weight—dry weight in A, and as the space of distribution of urea-C-14 in B and C. Preincubation (1 hr.) and incubation (1 hr.) were in balanced salt solution[3] with the additions as indicated. FFA and glucose were added during both phases, insulin and labeled tracers only during incubation.

*AIB, Sorbitol and urea spaces were calculated as $= \frac{\text{cpm/gm. tissue}}{\text{cpm/ml. final medium}} \times 100.$

†Calculated as $= \frac{\text{AIB space—Sorbitol space}}{\text{100—Sorbitol space}}$ where space $= \frac{\text{cpm/ml. tissue water}}{\text{cpm/ml. final medium}} \times 100.$

that octanoate exerted an inhibitory effect on AIB transport. The increased concentration of C-14 from AIB in the intracellular water of diaphragms incubated with 15 mM. β-hydroxybutyrate may reflect accelerated transport of AIB, or may be a result of decreased C-14 oxidation.

DISCUSSION

There is increasing evidence that fatty acids (or products of their metabolism) can affect various metabolic processes in vitro. They inhibit glucose utilization by muscle and fatty acid synthesis by the liver. The inhibitory effect of FFA on glucose utilization is exerted at several key metabolic steps: (a) inhibition of the transport of glucose across the muscle cell membrane, (b) deceleration of glycolysis via inhibition of phosphofructokinase and secondarily of hexokinase, (c) inhibition of pyruvate oxidation (for review of literature see references 1 & 2). In liver, long-chain acyl CoA derivatives competitively inhibit glucose-6-P-dehydrogenase.[17] Free fatty acids and long-chain acyl CoA derivatives inhibit hepatic acetyl CoA carboxylase. A negative feedback mechanism operating at this step has been suggested as a major site of regulation of fatty acid synthesis (for review of literature see references 18 & 19). Kalkhoff and Kipnis reported that the in vitro addition of 1 to 5 mM. palmitate to cell-free homogenates of rat liver did not affect protein synthesis, although FFA synthesis was inhibited.[18]

The data presented indicate that FFA, or byproducts of FFA metabolism can inhibit muscle protein synthesis in vitro. The inhibitory effect of FFA on the incorporation of C-14 from amino acids into muscle proteins was demonstrated with three different amino acids and three fatty acids, using "cut" or "intact" diaphragm preparations. The inhibitory effect of each FFA studied was evident when protein synthesis was stimulated by insulin; octanoate (1 mM.) caused significant inhibition also in the absence of insulin when glucose was omitted from the medium. The absence of glucose apparently potentiated the inhibitory effect of octanoate on protein synthesis, although octanoate was used as an energy source by the diaphragm (table 3). This may be due to the decreased availability of α-glycerophosphate for the esterification of FFA in muscle cells deprived of glucose.

Inhibition of protein synthesis by FFA was not secondary to inhibition of glycolysis, since it was demonstrated with or without glucose in the medium. The one-hour preincubation in a glucose-free medium assured that in experiments without glucose, this substrate was virtually absent from the extracellular space at the time the C-14 amino acid was added to the system and the muscle's glycogen stores were virtually depleted.[3]

Our data support the findings of Johnson et al.[20] and Manchester[13] indicating that diaphragms oxidize leucine and that C_1 is oxidized preferentially.[13] The present study suggests that leucine uptake and oxidation by striated muscle is influenced by fatty acids. Octanoate stimulated leucine uptake and oxidation while DL-β-hydroxybutyrate appeared to inhibit both parameters. β-hydroxybutyrate is an intermediate metabolite of leucine.[20] Therefore, the decreased C-14-O_2 production from leucine-U-C-14 during incubation with β-hydroxybutyrate may have been due to excess β-hydroxybutyrate competing with β-hydroxybutyrate-C-14 for oxidation. The decreased C-14 uptake from the medium during incubation with leucine-U-C-14 and β-hydroxybutyrate could be ascribed to isotope dilution only if a large proportion of the C-14 in the medium after incubation pertained to labeled breakdown products of leucine-U-C-14 which diffused out of the diaphragm. Thus, it is not clear whether or not the decreased uptake and oxidation of C-14 from leucine-U-C-14 in the presence of β-hydroxybutyrate represents inhibition of leucine oxidation by the ketone body. The increased uptake of C-14 from leucine-U-C-14 and its accelerated oxidation to C-14-O_2 during incubation with 1 mM. octanoate probably represents accelerated oxidation of the amino acid or of its fragments.

Whether or not the stimulatory effect of octanoate on leucine oxidation is related to its inhibitory effect on protein synthesis is not clear. Octanoate has been reported to uncouple oxidative phosphorylation of rat liver mitochondria in vitro.[21] Octanoate (1 mM.) inhibited the incorporation of C-14 from lysine-U-C-14 into proteins of diaphragms incubated with insulin, while the uptake and oxidation of C-14 from lysine were not affected by octanoate (unpublished results).

Although we have demonstrated that palmitate, β-OH-butyrate and octanoate can inhibit protein synthesis in muscle under certain in vitro conditions, the physiological significance of these observations is not established. The concentration of palmitate (and the palmitate/albumin ratios) used exceeded the range found in serum; the concentrations of β-OH-butyrate required to inhibit protein synthesis in vitro exceeded those seen in severe ketoacidosis. Octanoate was inhibitory at relatively low concentrations (0.5 mM.), however, octanoate is not a constituent of the mammalian FFA pool. The effectiveness of octanoate in inhibiting protein synthesis at low concentrations suggests that intermediate

chain length FFA may penetrate easier than other FFA into cellular compartments which are directly or indirectly involved in protein synthesis. Recent work indicates that octanoate infusions cause hypoglycemia in dogs, without increasing insulin secretion, probably by a direct inhibitory action on hepatic neoglucogenesis.[22]

Schonfeld and Kipnis studied the relationship between the extracellular and tissue fatty acid pools and the inhibition of glucose metabolism in "intact" diaphragms.[23] They found that the tissue fatty acid pool was not significantly influenced by increasing the palmitate concentration (or the albumin/palmitate ratio) of the incubation medium, while palmitate esterification by muscle increased with increasing medium FFA. They concluded that the tissue FFA pool is functionally heterogenous and that changes in glucose metabolism and insulin responsiveness correlate well with the tissue concentration of FFA but not with the extracellular concentration of palmitate.

The tissue FFA concentration in rat diaphragms is markedly influenced by several nutritional and hormonal factors. Increases of 200 to 400 per cent have been produced "in vivo" by fasting,[23,24] alloxan diabetes[24,25] and by the administration of epinephrine.[25] Epinephrine added in vitro increased the FFA content of isolated rat diaphragms severalfold.[25] The latter finding suggests that the epinephrine-induced increase in tissue FFA is a result of changes in the relative rates of tissue lipolysis/FFA utilization, rather than a reflection of the extracellular concentration of FFA.

Isolated rat diaphragms obtained from rats fasted for forty-eight hours incorporate less C-14 labeled amino acids into proteins than those from fed controls.[26] Epinephrine administered in vivo, or added in vitro to the incubation medium inhibits the incorporation of C-14 from amino acids into proteins in isolated rat diaphragms.[27] The results obtained in the present study with high concentrations of medium FFA suggest that protein synthesis in muscles may also be inhibited by increased intracellular FFA. It is tempting to speculate that intracellular FFA (or products of their metabolism) may have a regulatory influence on the rate of protein synthesis in muscle, or the rate of synthesis of certain muscle proteins. Such a mechanism would increase the supply of amino acids available for neoglucogenesis during stress or carbohydrate deprivation.

Manchester reported that insulin stimulates C-14 oxidation by rat diaphragms incubated with 1 mM. leucine-C-14.[13] In the present study, insulin did not stimulate C-14 oxidation from 0.05 mM. leucine-U-C-14. Similarly, during incubation with 0.05 mM. leucine-1-C-14, insulin failed to stimulate C-14 oxidation (unpublished results). The leucine concentration in the medium may be critical. As leucine in the medium is increased from 0.04 mM. to 1 mM. the C-14 fraction which is incorporated into proteins decreases from 40 to 10 per cent of the C-14 uptake.[13] When leucine in the medium is 0.002-0.06 mM., insulin stimulates its incorporation into proteins by 60 per cent; when leucine is 0.15 mM. the insulin effect is less than 20 per cent.[14] Since insulin increases the uptake of leucine from the medium (which implies accelerated penetration into the cell) and the rate of protein synthesis and since the intracellular amino acid pool is not homogenous,[10,11] it appears reasonable to postulate that at low extracellular leucine concentrations, the extra leucine that enters the cell under the influence of insulin is incorporated into proteins without becoming available for oxidation. At high extracellular leucine concentrations the protein synthetizing mechanism (although stimulated by insulin) may become saturated and more leucine becomes available for oxidation.

In the present study with 0.05 mM. leucine-U-C-14, insulin increased the uptake of C-14 from the medium, stimulated C-14 incorporation into proteins, did not affect C-14-O_2 production, and decreased the TCA soluble C-14 contained in the muscles. The latter finding suggests that either insulin accelerated protein synthesis more than it stimulated leucine transport, or that insulin promoted the incorporation of labeled breakdown products of leucine-U-C-14 into proteins[8] or lipids.

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loaded rats, induced by ureteral obstruction, and accompanied by an increase of H^3-inulin ratios without change in the Na^{22} ratios is very strongly suggestive that C^{14}-urea is trapped in the lumen of the distal tubule, together with H^3-inulin, while Na^{22} is being reabsorbed. This together with the rapid fall in the $^{TF}/P$ ratios of C^{14}-urea (and H^3-inulin) following release of the obstruction is strongly suggestive that urea is retained in the distal tubules because of their restricted permeability. These data suggest then that the restricted permeability of urea is adequate to explain increased distal tubular fluid urea concentrations at least under the conditions of ureteral obstruction.

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Role of Growth Hormone and Dietary Adaptation on Incorporation Of Leucine into a Mitochondrial Protein.* (32217)

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Nitrogen excretion studies on intact animals(1) led us to postulate that one function of pituitary growth hormone might be to speed the adaptation of the organism to the rapid utilization of fats to provide energy for protein synthesis under conditions under which little carbohydrate is available. Increasingly the metabolic effects of hormones are being sought at the subcellular level. Korner(2) has shown that incorporation of labeled amino acids into proteins of nuclei, mitochondria, microscomes and soluble fraction of liver was diminished by hypophysectomy and restored toward normal by growth hormone administration. In more recent work he has concentrated his attention on the growth hormone effect on protein synthesis in the ribosomal system. However, in the light of our observations on the effect of growth hormone on the nitrogen-sparing action of fat, we turned our attention to the well known protein synthesizing system of mitochondria. Since these organelles oxidize both carbohydrates and lipids, they might possibly be a site of adaptive changes which we postulated to occur after high fat feeding and after growth hormone administration.

Methods. Male mice of the C57/Fn strain, 3 to 5 months old, were fed either diet #343 containing 60% fat or diet #307 containing 60% carbohydrate(1) for at least 2 weeks prior to the experiment. Growth hormone,[1] 10 mg kg body weight, was injected intraperitoneally 5-5-1 2 hours before the animals were decapitated and exsanguinated. Several livers were pooled and the mitochondria isolated by the method of Truman and Korner (3). The incubation medium was essentially medium B of Truman and Korner using either succinate or octanoate and β-hydroxybutyrate as substrate. When using the latter substrate, the mitochondria were washed 4 times with 0.25 M sucrose and twice with 0.15 M KCl. Each ml of the incubation medium contained the mitochondria from 1 g of liver, 0.5 μc of

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[1] Growth hormone preparation NIH-GH-B8 Bovine was the gift of Endocrinology Study Section, NIH.

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TABLE I. Effect of Growth Hormone on Incorporation of Leucine-C^{14} into Protein in Isolated Mitochondria (Succinate Substrate).

Previous ration*	No. of trials	Growth hormone	Leucine-C^{14} incorporation, counts/min/mg protein/hr
High fat	(5)	—	151 ± 23
	(4)	+	320 ± 65
High carbohydrate	(4)	—	240 ± 42
	(5)	+	528 ± 63

* High fat ration consisted of 30% casein (Labco Vitamin-free), 55% hydrogenated fat (Crisco), 5% corn oil (Mazola), 5% starch, 5% salt mixture (Hubbell, Mendel, Wakeman).

High carbohydrate ration consisted of 30% casein, 60% starch, 5% salt mixture (Hubbell, Mendel and Wakeman), and 5% corn oil (Mazola).

Vitamins, with the exception of B_{12}, were intimately mixed with the casein prior to preparation of the rations.

leucine-C^{14}, and 1 mg of a synthetic amino acid mixture. The flasks were incubated in a metabolic shaker at 37° for one hour under an atmosphere of air. The reaction was stopped by adding $HClO_4$ containing unlabeled leucine, and the protein was isolated as directed by Truman and Korner. The dry protein thus obtained was dissolved in 0.5 N NaOH. The nitrogen content of this solution was measured by the method of Johnson(4). The C^{14} content was determined by liquid scintillation counting of 0.2 ml of the alkaline protein solution in 10 ml of Bray's solution (5).

Results and discussion. When succinate was used as substrate we found that mitochondria from fat-adapted and carbohydrate-adapted animals incorporated labeled leucine into mitochondrial protein to about the same extent. Treating mice, regardless of their dietary history, with growth hormone produced an increase in the degree of *"in vitro"* incorporation of amino acid (Table I). These results confirm the observations of Korner(2).

On the basis of our studies with intact animals(1) we suspected that, when octanoate was used as substrate, mitochondria from animals adapted to high fat rations would incorporate labeled amino acid into protein to an extent not affected by previous growth hormone treatment. Mitochondria from carbohydrate-adapted mice would be expected to incorporate amino acid to a lesser degree, and previous growth hormone treatment would be expected to increase incorporation to the level observed in the fat-adapted animals. The results are shown in Table II and are compatible with our suggestion that growth hormone may act in part by bringing about adaptive changes which facilitate the utilization of lipids to provide energy for protein synthesis and that these adaptive changes can occur without growth hormone administration if the animal is given time to adapt itself to a high fat ration.

Mice fed a high fat ration *ad libitum* consume more calories than those fed a high carbohydrate diet. It is difficult to say whether a small difference in caloric intake prior to the experiment would drastically affect the reaction of mitochondria to growth hormone.

In our studies with intact animals(1) carbohydrate-feeding gave a maximal nitrogen-sparing action which could not be increased by growth hormone administration. The pronounced effect of growth hormone when succinate was used as substrate (Table I) is not in harmony with the earlier findings and suggests an additional mode of action of growth hormone than the one we have already proposed.

Summary. Isolated mitochondria from animals previously treated with growth hormone incorporated more amino acid into protein when succinate was substrate. In the presence of β-hydroxybutyrate and octanoate the mitochondria from fat-adapted animals incorporated amino acid at a greater rate than mitochondria from carbohydrate-fed ones. In this latter study, growth hormone enhanced incorporation rate only in mitochondria from

TABLE II. Effect of Growth Hormone on Incorporation of Leucine-C^{14} into Protein in Isolated Mitochondria (Octanoate Substrate).

Previous ration*	No. of trials	Growth hormone	Leucine-C^{14} incorporation, counts/min/mg protein/hr
High fat	(4)	—	294 ± 37
	(5)	+	280 ± 25
High carbohydrate	(3)	—	171 ± 23
	(5)	+	287 ± 26

* Composition of rations as indicated in Table I.

animals fed a high carbohydrate ration.

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Healing of Urinary Bladder Wounds. Uptake of S^{35}-Sulfate in Acid Mucopolysaccharides.* (32218)

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Wound healing processes involve biochemical and morphological changes usually in the connective-tissue elements of the tissue. The importance of collagenous fibers for tensile strength is well-documented. The fibers and cells are embedded in the amorphous ground substance, containing acid mucopolysaccharides, electrolytes, hormones, and many other substances. The acid mucopolysaccharides are polysaccharides containing hexosamine and uronic acid. They are present as macromolecules, linked to proteins. Even if the exact mode of action is unknown, they undoubtedly participate in biologic processes such as inflammation, tissue repair and ageing.

The aim of this study was to examine the synthesis of sulfomucopolysaccharides in healing linear wounds in the urinary bladder of rabbits, by measuring the uptake of $S^{35}O_4$. The results of biochemical and morphological studies on mucopolysaccharides and collagen in the same wounds have previously been reported(1,2).

Material and methods. Fifty-five male albino rabbits weighing about 2.5 kg were kept on an adequate laboratory diet for at least one week before operation. Five animals were not operated, but were killed at the time of operation and served as controls. The animals were anesthetized by intravenous injection of 60 mg Nembutal® supplemented by ether inhalation. The abdomen was opened through a low midline incision. The urinary bladder was emptied by puncture and aspiration. An incision through all layers was made in the midline of the anterior wall from the top to the neck. The bladder was closed in 2 layers with continuous sutures of 4-0 nontraumatic silk. In the first layer the suture was passed through the entire bladder wall near the wound edge. The second suture was seroserous. The abdominal wall was closed in 2 layers with silk sutures.

The operated animals were divided into 11 groups, sacrificed 2 to 28 days after the operation (Table 1). They were killed by intravenous injection of 300 mg nembutal. Forty-eight hours before sacrifice the rabbits were given an intraperitoneal injection of 1.5 mCi $S^{35}O_4$ in aqueous solution. The original carrier free S^{35}-labelled sodium sulfate obtained from the Radiochemical Centre, Amersham, England, was diluted by adding 0.04% sodium sulfate to a final activity of 1 mCi per ml. The wounds were removed, and, after drying and defatting, the tissue was homogenized in 0.5 N NaOH. The uptake of $S^{35}O_4$ was determined by the method of Moltke(3) as modified by Marckmann(4). After wet ashing of 2.5 ml homogenate with fuming nitric acid in a sand bath, the $S^{35}O_4$ was precipitated as barium sulfate. The samples were counted at infinite layer thickness with a Geiger-Müller tube to a statistical error below 3%.

In 2 animals the ureters were ligated through an abdominal incision before the injection of radiosulfate. The animals were

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A spray sequence specific for the detection of free fatty acids

A sequence of spray reagents used after the separation of a lipid extract, into classes, by thin-layer chromatography revealed unexpectedly the position of free fatty acids as a rose-violet spot on a pale yellow background. Subsequent tests showed that no other major class of lipids responded similarly, nor did some other types of carboxylic acids.

The reagents were (a) 2′,7′-dichlorofluorescein, a general lipid indicator, (b) alcoholic aluminium chloride, a flavanoid indicator and (c) acid ferric chloride, a phenol and flavanoid indicator. The colour response was obtained only when all three reagents were used, and although the order of spraying was not important, it was most useful to visualise all lipid classes first with (a) under U.V. light. Sprays (b) and (c) could be combined but results were more reproducible if they were used separately, and the colour was brighter if (b) preceded (c). It was not necessary for the ferric chloride solution to be acidified, but it had to be aqueous and not alcoholic.

The response of lipid mixtures and the sensitivity of the fatty acids were tested on Silica Gel G plates which had been developed with petroleum ether–diethyl ether–acetic acid (80:20:1) for neutral lipids and chloroform–methanol–water (60:30:1) for polar lipids. The fatty acids linolenic, linoleic, vaccenic, oleic, stearic, palmitic, capric and caprylic could be detected at the 2 μg level; with caproic, *n*-valeric, isovaleric, *n*-butyric and isobutyric acids, increasingly higher amounts were needed; and with propionic and acetic acids, there was no detectable response. Negative results were obtained with cholesteryl oleate, methyl oleate, triolein, cholesterol, phosphatidylethanolamine, lecithin, sphingomyelin, cerebrosides and sulphatides and also with citric, oxalic and lactic acids. The reaction, as a simple spot test, has been carried out on filter paper, with the same results as were obtained on Silica Gel G plates. However, for those compounds giving a positive response, the sensitivity was approximately halved.

Acetic acid in the developing solvent caused no interference with colour development, as happens with most indicator sprays used for detecting organic acids. However, alkali did interfere, and the sequence did not work over a Rhodamine-6-G spray when the latter was made alkaline. The sequence gave a positive result when applied over an antimony trichloride spraying or after the plates had been exposed to iodine.

The reaction has proved useful in checking the efficiency of column chromatography for separating free fatty acids from triglycerides and polar lipids.

Method

The reagents are: (a) 0.05% 2′,7′-dichlorofluorescein in 95% ethanol, (b) 1% aluminium chloride in ethanol, and (c) 1% aqueous ferric chloride.

The plate (or paper laid on a plate) is sprayed to dampness with each reagent in turn and heated for a few minutes after each spraying. This heating, which is essential, may be done in an oven but it is satisfactory if the plates are laid on top of a closed boiling waterbath. On paper the spots are permanent; on thin layer plates they soon fade, though a further spraying with (c) will restore the colour.

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In the present work a qu...
described for the separation and
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applied to the study of the struc...
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Materials and methods

Hydrolysis of the samples. ...
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200 μl 2 *N* HCl at 100° for 1.5–3 h...
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was dissolved in 20 μl of water.

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Scientifique et Technique), No. 66 00...
** See "Note added in proof" on ...

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Major Free Fatty Acids of Feta Cheese[1]

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Abstract

A determination of the major free fatty acids (FFA), acetic through oleic, was carried out in ten samples of imported Feta cheese. Representative variants of Teleme and also mild- and sharp-flavored Feta were analyzed, using liquid partition and gas-liquid chromatography. A low total FFA value characterized all Teleme samples. The dominant acid (25–46%) was acetic. In the mild-flavored Feta samples the FFA varied considerably, but their total value was generally low. Again, the acetic acid comprised a high portion of the total acids (28–43%). In the sharp-flavored samples, moderate to excessive amounts of FFA were detected. The acetic acid content did not change but its ratio to the total FFA showed a sharp decrease (5–12%). The butyric acid content increased and its weight percentage was more than four times the reported average for the esterified acid. Increased levels of caproic, caprylic, capric, lauric, myristic, palmitic, and oleic acids also were encountered. With the exception of palmitic and stearic, found in lower percentages, the relative concentrations of all other acids approximated the ratios of their esterified counterparts in the milk fat. Relevance of the data to the flavor of the cheeses is discussed.

Feta cheese has come recently under technological and analytical scrutiny and efforts have been made to relate its organoleptic characteristics to manufacturing practices as well as to data of chemical composition. The free fatty acid composition, critically affecting the flavor of this cheese variety (5), has not yet been reported. This is particularly true of sheep or goat milk Feta cheese, the predominant type consumed abroad and in this country. Recent analytical studies on Greek Feta cheese have included the determination of only the lower fatty acids, collectively expressed as per cent volatile acidity (9). Quantitative information is also lacking on the individual, less volatile, long-chain members of the FFA spectrum. The present study was undertaken in an attempt to fill the existing gap by determining the FFA profile of some representative variants of imported Feta cheese.

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Experimental Procedure

Selection of cheese for analysis. Ten samples of cheese, representing currently imported Feta variants, were analyzed for their FFA content. In selecting the samples, consideration was given to their geographic origin. Samples from cheese lots imported from four countries, Italy, Greece, Rumania, and Bulgaria, were included in the study. A Feta cheese type, distinct on manufacture grounds as well as geographic origin, is the so-called Teleme cheese, extensively produced in Rumania, Bulgaria, and Northern Greece (4, 12). Three samples of this type were included in the study. Samples were also selected on the basis of their mild or sharp flavor, indicated by their retail label. The mild label stood for cheese with little or no detectable rancidity, whereas a sharp Feta was cheese more or less with pronounced flavor, due to extensive lipolytic activity. All samples were obtained from stores in the local New York market and were at least four months old. A summary of the major organoleptic characteristics determined by a consumer panel examination prior to chemical analysis, along with the origin and measured acidity of the samples, is given in Table 1.

Determination of acetic, propionic, and butyric acids. Acetic, propionic, and butyric acids were resolved and quantified by the silicic acid column chromatography procedure of Keeney (8). Duplicate samples were used for all analyses. The total mole equivalent of all FFA higher than butyric was also determined by the same method. Two 10-g samples of each cheese were weighed in 100-ml beakers and acidified with a few drops of a 50% sulphuric acid to a pH 2.0 ± 0.2. About an equal amount of dried silicic acid (170 C for 72 hr) was added and the sample was ground and mixed to a homogeneous, nonlumping powder. The prepared sample was transferred in a little hexane to the top of the packed column and eluted with n-butanol in hexane. Since only one acid, acetic, consistently showed a distinct yellow band on the green column, it was neces-

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TABLE 1

Descriptive summary of Feta cheeses selected for analysis

Cheese no.	Country of origin	Quality characteristics[b] Flavor	Body and texture	pH
1[a]	Rumania	Acid (slight), salty (moderate)	Firm, soluble (moderate)	4.75
2[a]	Bulgaria	Acid (pungent), flat	Crumbly, granular	4.18
3[b]	Rumania	Acid (pungent), salty	Firm, soluble (moderate)	4.47
4	Italy	Acid, salty (moderate), bitter (slight)	Firm, soluble (moderate)	4.71
5	Italy	Unnatural, fermented, atypical odor	Soft (moderate), soluble (pronounced)	5.21
6	Italy	Acid (slight), semi-ripe	Firm, soluble (moderate)	5.10
7	Greece	Atypical odor	Firm, soluble soluble (slight)	5.28
8	Greece	Ripened, salty (slight)	Soft, soluble (pronounced)	4.56
9	Greece	Ripened, rancid (slight)	Firm (moderate), crumbly, soluble (moderate)	5.00
10	Italy	Rancid (pronounced)	Firm, insoluble	4.73

[a] Composite sample.
[b] Obtained by consumer panel examination as in (5).

sary for a quantitative recovery of the acids to calibrate the volumes of the respective acid fractions by first resolving known varying mixtures of short-chain FFA under identical conditions of analysis. With a flow rate of 3.4 ml/minute, four elution fractions were collected, 105, 120, 175, and 300 ml, respectively containing the fractions of higher than butyric, butyric, propionic, and acetic acids. The first three fractions were eluted, using 1% n-butanol in hexane, whereas the acetic acid was recovered from the column with an eluant of 5% n-butanol in hexane.

Determination of caproic through oleic FFA. Individual FFA higher than butyric were identified and their mole percentages determined by gas-liquid chromatography. Quantitatively, they were estimated by distributing the total moles of higher fatty acids determined by titration of the respective fraction from the Keeney [illegible]

FFA in mixture with the unsaponified [illegible] were separated from each cheese sample by [illegible] method reported by Bills and Day (1). [illegible] to use 360 g of Feta cheese, to secure, [illegible] centrifugation, a sufficient quantity of [illegible] for analysis (about 30 g). The cheese [illegible] were ground with a mortar and pestle [illegible] with 50% sulphuric acid to a pH [illegible] The samples were packed into [illegible] stainless steel centrifuge tubes warmed [illegible] a 40 C water bath for 30 min, and centrifuged in a B-20 IEC superspeed centrifuge for 20 min at 30,000 × *g*. The separated fat, forming the uppermost layer in the tubes, was drawn off carefully after centrifugation with a pipette fitted with a propipette.[2] Isolation of the FFA from the unsaponified fat and their esterification for GLC was carried out according to a modification of the method of Bills, Khatri, and Day (2). The separated fat-FFA mixture was added to a conditioned anion base exchange resin (Amberlite 400). After adsorption, the unsaponified fat was removed from the resin by repeated washings with hexane, absolute ethanol, and methanol. The fatty acids were then esterified directly on the resin by a HCL-methanol mixture and extracted with ethyl chloride at 2 C. The ethyl chloride extract was concentrated to a volume of 4 ml and carefully transferred to a screw-cap tube and stored at −20 C. A final condensation to approximately 1 ml was carried out just prior to injection of the mixture into the gas chromatograph.

Analysis of the fatty acid methyl ester mixtures was carried out by means of a gas chromatograph (Perkin-Elmer Model 154D Vapor Fractometer fitted with a Flame Ionization Detector and a Leeds Northrup Recorder). A Perkin-Elmer 200-cm stainless steel column, of ¼-in. outside diameter, packed with 20% diethylene glycol succinate polyester on 60- to

[2] Pipet filler made of rubber and having three agate ball valves. Obtained from Fisher Scientific Company, New York.

80-mesh GC-22 support material, was used to resolve the mixture of esters. The column was operated isothermally at 196 C. Nitrogen, the carrier gas, was used at a pressure of 35 mm Hg. Conditions of operation of the flame detector were 12 psi hydrogen and 30 psi air. The individual fatty acid methyl esters from the cheese samples were identified by a comparison of the retention times of the unknown esters with those of known standards.[3] The molar ratios of the esters were determined by comparing the areas under the peaks drawn by the recorder. The component acids were then estimated quantitatively by distributing the titration value of the higher fatty acid fraction from Keeney's column, according to the molar ratios of the methyl esters of all acids higher than butyric, obtained from the GLC chromatograms.

With the GLC method applied in this study, distinct peaks were obtained for the following acids: 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, 18:0, and 18:1. Linoleic (18:2) and linolenic (18:3) acids could not be detected in any of the samples analyzed. FFA composition of the ten samples is given in Table 2.

Results and Discussion

The analyzed samples represented Feta cheese variants differing substantially as to their manufacturing method, age, flavor, body, and texture characteristics. For a meaningful comparative study of their FFA composition, they were selected, organoleptically examined, and analyzed in three groups, each one sharing a common major characteristic considered likely to affect the FFA content of the cheese in the same way.

In the first group, three samples of the same type, Teleme, were included (no. 1, 2, and 3, Tables 1 and 2). Manufacture of this truly pickled cheese type differs considerably from that of common Feta in this respect: Early in the curing process Teleme is subjected to salting in a brine solution of 12–15% salt concentration for 24 hr (4) or even 22–24% for 16–18 hr (12). This practice evidently interrupts the biochemical activities in the ripening cheese and primarily the utilization of lactic acid, accumulated by the initial fermentation of the starter culture. Consequently, the acidity of the resultant Teleme cheese remains high for a long time, especially if it is refrigerated soon after manufacture (12). Common Feta, on the other hand, is typically dry-salted and never in its ripening process does it come into contact with similar high salt concentrations. The titratable acidity of Greek Teleme after five months of ripening was reported as high as 1.34% (expressed as lactic acid), whereas the highest values reported for common Feta, ripened for the same period, were below 0.9% (9). Similar high acidity values have been reported for ripened Rumanian Teleme (4).

All three samples of Teleme analyzed demonstrated low pH values (Table 1). Furthermore, on organoleptic examination their curd creaminess or curd solubility (5) was found less than pronounced and in one case (Sample 2) completely lacking. On the other hand, in comparison with the rest of the analyzed cheeses total FFA values of the Teleme samples were low (Table 2). This low FFA content in the presence of high acidity most probably ex-

[3] Qualitative mixtures L-201, L-205, and L-206 obtained from Applied Science Laboratories, University Park, Pennsylvania.

TABLE 2

Free fatty acids in 10 samples of Feta cheese[a]

Cheese no.[b]	2:0[c]	3:0[c]	4:0[c]	6:0[d]	8:0[d]	10:0[d]	12:0[d]	14:0[d]	16.0[d]	18:0[d]	18:1[d]	Total FFA[c]
1	275	33	79	3	10	22	21	57	195	86	277	1,058
2	692	30	76	2	5	27	27	78	204	98	236	1,475
3	669	43	150	3	8	31	59	89	285	119	410	1,866
4	416	32	80	3	5	19	20	56	141	62	177	1,011
5	868	50	123	9	21	77	57	145	363	187	524	2,424
6	545	36	82	7	10	40	36	80	169	47	192	1,244
7	605	83	174	10	15	61	54	166	387	135	412	2,182
8	492	87	824	43	134	615	320	308	458	189	755	4,225
9	521	167	651	37	73	301	201	353	818	435	1,278	4,835
10	528	276	1,778	282	422	1,494	1,194	1,103	1,839	274	1,686	10,876

[a] Results reported as milligrams free fatty acids per kilogram cheese (wet basis).
[b] Refer to the same samples as in Table 1.
[c] Average values of duplicate analyses according to Keeney's column chromatography technique (8).
[d] Data obtained by GLC.

emplifies the well-known inhibitory effect that extremely low pH values have on the action of lipases on milk fat. Quantitatively, the predominant acid present (25–45%) was acetic. The volatile fatty acid (VFA) values (mainly acetic acid, Table 2) were in agreement with the low volatile acidity reported for Greek Teleme cheese, ripened for five months (0.05–0.08% expressed as butyric acid) (9). In view of the established low total FFA values and the preponderance of acetic acid, it seems then unjustified to express volatile acidity in Teleme as butyric acid. Because of this consideration, and also due to the substantive contribution of acetic acid to a pickled, harsh, but not rancid flavor and a related typical aroma generally characterizing Teleme, in my opinion the expression of volatile acidity on an acetic rather than butyric acid basis would be more valid as well as expedient.

The next group of four samples (no. 4, 5, 6, and 7, Tables 1 and 2) represented common, mild-flavored Feta cheese. Their pH values were generally higher than those of the Teleme samples. The acidity of Feta cheese produced in various cheese-making districts throughout Greece and ripened for the same periods, has been reported in a recent survey study cited above (9). In cheese ripened for five months, it has been found to range between 0.5 and 0.8%, expressed as lactic acid.

Total FFA values of the four samples varied between 1,011 and 2,424 mg per kilogram cheese. They were comparable in two cases (Samples 4 and 6, Table 2) with those of the Teleme samples, whereas in the other two samples (5 and 7) they were nearly twice as high. In comparing the overall flavor of the Teleme and the mild Feta samples, substantial differences were noted even when samples equivalent as to their FFA content were compared. It is obvious that the role of FFA in determining the quality and intensity of flavor in nonrancid Feta and Teleme cheeses is rather limited. Additional factors such as total acidity and degree of protein degradation are also of great importance, so that when the FFA content of different samples is the same, the latter factors appear to be the decisive ones in determining flavor. The acetic acid comprised 28–43% of all FFA present in the mild samples of Feta, i.e., it varied within a similar range and likewise constituted the most important single acid fraction as in the Teleme samples. These data indicate the importance of acetic acid as a major fatty acid constituent and as a flavor and aroma determinant in Feta cheese. A similar role for acetic acid has been recognized in Cheddar (11) and, indeed, numerous other cheese varieties. Nevertheless, the metabolic origin of acetic acid in cheese ripening is still uncertain. The subject has been under investigation, but conclusions in the literature are conflicting. Nakae and Elliott (10) have presented evidence by which the production of acetic acid in cheese is explained by the enzymatic behavior of lactic acid bacteria, probably by processes of oxidative deamination or decarboxylation of amino acids released in the course of ripening from cheese proteins. On the other hand, Budslawski and Rozowicz (3), who recently studied the development of volatile fatty acids in the ripening process of Tilsit cheese without discounting the same possibility, at least for advanced stages of cheese ripening, contended that in the initial stages of ripening acetic acid was primarily produced by lactic fermentation.

For the elucidation of this metabolic and ecology problem of cheese biochemistry, and in view of the distinctive position that acetic acid has in the FFA spectrum of the nonrancid Feta cheese (Samples 1 through 7, Table 2), this type of cheese may lend itself as a very suitable study material.

In Samples 8, 9, and 10 (Tables 1 and 2), selected for their varying rancidity, pH values determined did not establish a definite pattern. They varied between 4.56 and 5.00 (Table 1). The variation in acidity may or may not reflect different stages of ripening of the three samples. Such wide variation has been reported for Greek Feta cheeses ripened for the same period (9), although the maximum titratable acidity determined in those cheeses was much lower than that reported for Teleme.

Available evidence from other experiments (6) seems to support the observation that ripening proceeds at a higher rate in common Feta than in Teleme. This difference has been reflected not only in the disparity of acidity levels in Feta and Teleme but in the degree of lipolytic activity and protein hydrolysis, as well. In this respect, total FFA determination and nitrogen distribution studies have both yielded higher values in Feta than in cheese ripened for the same period under Teleme-like conditions.

Total FFA values of the same sharp-flavored samples varied between 4,225 and 10,876 mg per kilogram cheese. Thus, they proved consistently higher and in one case (Sample 10) far exceeded FFA values of all other samples. Amounts of acetic acid determined in these samples appeared comparable to those of the nonrancid samples. However, the quantitative

ratio of acetic acid to the total FFA was sharply decreased to a low 5–12% range. Free butyric acid was detected in considerable amounts, making up 13–19% of the total FFA. Such high percentages indicated selective lipolytic activity in the cheese, since they appeared much higher than the average figures cited by Hilditch and Williams (7) for butyric acid in the sheep and goat milk fats. Concentrations of all FFA higher than butyric also appeared increased. Their relative proportions, however, were in general agreement with the average figures of Hilditch and Williams, given for each fatty acid component of the sheep and goat milk fats. But the percentages of free palmitic and stearic acids were somewhat lower than the values cited by these authors. The high amounts of total FFA determined in the last three samples could be associated with the type of rancid flavor sensed in these cheeses. In Samples 8 and 9, where the total amount of FFA was about twice as much, in comparison with the nonrancid samples, the intensity of flavor was moderate and its quality typical of full ripened Feta. Efthymiou and Mattick (5) have described typical Feta flavor as pleasing and acetic-rancid. The FFA determination of Samples 8 and 9 substantiated the organoleptic evaluation of their flavor and produced an analytical profile essentially conforming to the given definition of typical Feta flavor. In Sample 10, the total FFA level was almost five times that of the mild-flavored samples; flavor in this cheese was objectionably rancid, peppery, and soapy. This type of flavor has been associated with high levels of long-chain fatty acids in experimental domestic Feta cheese (5). It is noted that a similar analytical profile was revealed in this very rancid sample. In view of the data obtained in the present study (Table 2), there seems to be no dependence of the objectionable rancidity on a specific fatty acid component but rather on a minimum threshold concentration, as yet undetermined, of the total FFA except acetic. In addition, it can be suggested that a perceptible impact on rancid sensation is primarily exerted by the relatively high concentrations of free butyric, caprie, lauric, myristic, palmitic, and oleic acids.

Traditionally, Greek cheese-makers have effected lipolysis in cheese through use of rennet paste from lambs and kids (12). More recently, commercial preparations of pregastric esterases were tried as flavor-inducing agents in Feta cheese made from pasteurized cow's milk (5). Certain of these preparations, also of lamb and kid origin, were found to favor development of typical Feta-like flavor.

Acknowledgment

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THE ABSORPTION OF FATS STUDIED IN A CHILD WITH CHYLOTHORAX

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The way in which fat is absorbed in healthy people is still largely unknown. This lack of knowledge chiefly concerns the question whether complete hydrolysis of the dietary fat is necessary before it is absorbed, or whether absorption through the intestinal wall is possible without complete hydrolysis. In the past few decades this problem has been studied mainly by Verzár and McDougall (1, 2) and by Frazer (3–12), although in widely different ways.

The quintessence of Verzár's theory is: complete hydrolysis of fat in the intestine and synthesis of the fatty acids to triglycerides in the intestinal cells, *via* an intermediate stage of phospholipids. The main features of Frazer's theory are: hydrolysis of part of the fat into mono- and diglycerides and free fatty acids in the intestine, formation of an emulsion and absorption of tiny droplets of fat. The essential point of difference between these two theories is the degree of hydrolysis of the fat in the intestine. This problem has been approached in various ways.

The degree of hydrolysis was determined directly by examination of the intestinal contents after administration of fat by Mattson, Benedict, Martin, and Beck (13), Desnuelle and Constantin (14) and Kuhrt and other workers (15). Other attempts to determine the degree of hydrolysis using an indirect method of calculation, by comparison of chyle fat with dietary fat labelled with isotopes, were made by Borgström (16), Reiser, Bryson, Carr, and Kuiken (17, 18) and Bernhard, Wagner, and Ritzel (19). All these investigations led to the conclusion that there is a considerable hydrolysis of fat in the intestinal lumen. According to Frazer (10) about 40 per cent of the dietary fat is hydrolyzed partially, but Borgström and Reiser consider it highly probable that the hydrolysis, in any case into mono- and diglycerides, is practically complete. The latter authors assume that 25 to 45 per cent of the dietary fat is completely broken down into free fatty acids and glycerol; the remaining 55 to 75 per cent is, according to them completely, and according to Bernhard partially, hydrolyzed into monoglycerides.

There is, however, quite another aspect to this problem. Frazer (6–9), Bernhard (20), Borgström (21, 22), Bloom, Chaikoff, and Reinhardt (23) and Favarger (24) have shown that not all fatty acids are absorbed in the same manner.

According to Frazer the fatty acids of low molecular weight are absorbed in a different manner from those of high molecular weight. Frazer also deals separately with the unsaturated fatty acids, such as Δ^9-octadecanoic acid. As the dietary fats may be of widely varying composition, the determination of the individual absorption of all fatty acids, of which the dietary fat concerned is composed, is of great advantage, in contrast to the determination of the total absorption of fat or of the absorption of one particular labelled fatty acid.

The method of Boldingh (25, 26) enables such an analysis. A case of chylothorax in a child offered us a welcome opportunity to use this method in order to study the absorption of the separate fatty acids (27).

CLINICAL FEATURES

Chylothorax is a rare anomaly, arising when the pleural cavity of one or both lungs fills with chyle. The anomaly is caused by a lesion of a chyle vessel. The patient who gave us the opportunity of conducting this investigation was the third child of healthy parents; he was born on July 13, 1950. The other children were healthy. The mother's pregnancy was uneventful; parturition was normal. Directly following birth it was noticed that the patient had a swollen abdomen and a bilateral testicular hydrocele. When the boy was two months old, operation on a bilateral inguinal hernia (which had developed gradually) revealed that the swollen abdomen was due to chyleascites. Seven months later, a right-sided chylothorax was found in addition.

Cap Acid

The signs and symptoms of chylothorax and chyloascites alternated in predominance, without any operation being necessary, up to the age of nearly two years. Then he began to have attacks of dyspnea until he was admitted to the hospital, because of the severity of the dyspnea, at the age of 23 months.

On examination no other abnormalities were found apart from a barrel-shaped thorax and much fluid in the right pleural cavity. There was no fluid in the abdominal cavity. Feces, urine and blood were normal on morphological and chemical examination. Liver function and fat balance were also normal (the latter had been disturbed only for a short period during and after an infection). Although the patient's nutritional condition was not optimal, his growth during his affection and after recovery from it was always normal. On the strength of these data the patient was regarded as a healthy child with a purely anatomical abnormality. It may therefore be assumed that the chyle of this patient was also normal.

The right pleural cavity was drained weekly by aspiration during his eight months' stay in the Pediatrics Department. The secretion of chyle into the pleural cavity diminished during the last few weeks of this period and had ceased completely at the end of the eight months. So far relapses have remained absent and, at the time of writing, the child is in good condition.

The patient's chyle came up to the requirements of Baldridge and Lewis (28): 1) its color was that of milk; 2) a cream-like upper layer separated from the fluid at rest; 3) it was an emulsion of fine fat droplets; 4) its reaction was alkaline; 5) the fluid was sterile and not liable to decomposition; 6) the fat content was usually high, varying between 0.4 and 4.0 per cent; 7) the protein content was usually more than 3.0 per cent; 8) the fluid contained many lymphocytes. It appeared moreover that: the fat content of the chyle was dependent on the fat content of the diet; and, the fatty acid composition of the chyle fat was dependent on the fatty acid composition of the dietary fat. These relationships are characteristic of chyle and constitute proof that the fluid aspirated from the thoracic cavity was actually chyle. Results of laboratory examinations are shown in Table I.

TABLE I

Some characteristics of the patient's chyle

	Number of determinations	Range	Average
Cells (mainly mononuclears)	18	(900–6000)	3000/mm³.
Protein (Kjeldahl)	6	(3.37–4.57)	4.00 gm. %
Albumin	6	(1.79–2.54)	2.25 gm. %
(α+β)-Globulin	6	(0.14–0.60)	0.50 gm. %
γ-Globulin	6	(0.77–2.31)	1.25 gm. %
Cholesterol	25	(90–230)	130 mg. %
Cholesterol esters	25	(60–220)	65 mg. %
Potassium	6	(3.7–4.2)	4.0 mEq./L.
Sodium	6	(137–141)	140 mEq./L.
Calcium	6	(4.5–4.8)	4.7 mEq./L.
Chloride	6	(99–109)	103 mEq./L.
Phosphorus	6	(4.9–5.1)	5.0 mg. %

EXPERIMENTAL

Aim of the investigation

The aim of the investigation was to study the relationship between dietary fat and chyle fat, to arrive at conclusions on the absorption of fats in man, and to test these conclusions against the current theories on the mechanism of fat absorption.

Arrangement of the experiments

On the strength of animal experiments and based on Munk's investigations on a patient with a chylous fistula, it is to be expected that a great deal of the fat, under normal circumstances, is absorbed with the chyle. It is, therefore, possible to study the fat absorption, at any rate that via the lymph, by comparison of the composition of chyle fat with that of dietary fat. In contrast to former investigations, it is now possible to compare the composition of the chyle fat as a whole with that of the dietary fat, by means of separate quantitative determinations of all fatty acids. As not all intestinal chyle accumulates in the patient's thorax, absolute measurement of the absorption via the lymph was not possible.

The natural fats, which are mixed triglycerides being composed of widely varying proportions of the fatty acids, n-octanoic to n-docosanoic, were administered to the patient in such a way that each time certain particular fatty acids could be studied. When the patient was given butterfat, the emphasis fell on the short-chain fatty acids, n-tetranoic, n-hexanoic, and n-octanoic; when given margarine and coconut oil, n-octanoic, n-decanoic, and n-dodecanoic acid were preponderant. A synthetic fat, "capryl olive oil," was administered to study the behavior of n-octanoic acid more closely; cacao-butter was given to examine that of n-hexadecanoic and n-octadecanoic acid; and finally a synthetic fat with hydrogenated herring oil was given to investigate the absorption of n-eicosanoic and n-docosanoic acid.

The next step was to study the absorption via the lymph of some *unsaturated fatty acids*. By feeding sunflower oil and linseed oil the absorption of $\Delta^{9,12}$-octadecadienoic acid and $\Delta^{9,12,15}$-octadecatrienoic acid was investigated, while the behavior of Δ^{13}-docosenoic acid was studied by the administration of rapeseed oil. Comparison of the lymphatic absorption of olive oil and cladinized olive oil enables us to investigate the absorption of Δ^{9}-octadecenoic acid (both cis- and trans-isomers).

As the lymph was aspirated only once a week, the possibility could not be excluded that some fatty acids might be absorbed from the thoracic cavity to a greater extent than others. This is, however, unlikely because the fatty acids are almost completely present as fat in the lymph. A differentiation is therefore impossible here and a specific absorption can therefore not be expected.

It is possible that part of the fat is absorbed via the portal system and the liver. This was not investigated in the case concerned. The possibility should not be overlooked that some fat escapes absorption and is excreted in the feces. In order to keep a check on this the fat balance was always determined by the method of van de Kamer (29).

The patient was kept on a diet with constant amounts of fats, proteins, and carbohydrates during most of the experiments. The daily diet consisted of:

200 ml. protein milk [1]	75 gm. green vegetable
20 gm. rice flour	25 gm. lean meat
20 gm. sugar	16 gm. fat
400 ml. water	75 gm. banana
50 gm. potato	50 ml. orange juice
100 ml. tomato juice	

Vitamins A, D, B-complex [2] and C were added to this diet. Stabilized ferrous chloride was also given per os. The amount of fat was 28 gm. per day; exceptions were made during some periods in which larger or smaller amounts were given. It was necessary during the last two months to increase the amounts of protein and carbohydrate in order to meet the increasing caloric demands.

The type of fat in the diet was changed every week, or group of weeks, by giving a protein milk and fat containing different kinds of glycerides, such as coconut oil or olive oil instead of butterfat.

The chyle was collected by pleural puncture at the end of each week, and subsequently the pleural cavity was examined radiologically for the presence of chylous residues. Because of the possibility that a slight residue may influence the composition of the chyle during the following dietary period, a diet containing one kind of fat was usually given for two successive weeks. The composition of the chyle fat during the second week was considered the most representative.

Methods

The chemical investigation was carried out as follows: All saturated fatty acids with the exception of n-tetranoic acid were quantitatively determined separately. Of the unsaturated fatty acids, only the poly-unsaturated ones could be determined individually by measurement of the absorption spectrum in ultraviolet light after alkaline isomerization. It was impossible to determine the mono-unsaturated fatty acids directly, either separately or *in toto*. The sum of the unsaturated fatty acids was computed by subtracting the total of the saturated fatty acids from the total of all fatty acids. The iodine number also lent support to this calculation. With these restrictions the fatty acid composition of each dietary fat could be compared with that of the corresponding chyle fat. The extraction and analyses of the fat from chyle, feces and blood were carried out by the methods of van de Kamer and his colleagues (30), using the analytical method of Boldingh (25, 26). A mixture of the analytically pure saturated fatty acids, n-hexanoic to n-docosanoic, which contains of each fatty acid 2 to 8 mg., can be analyzed with this method so that each acid is estimated with an accuracy of about 5 per cent. The minimum amount which can be determined with this accuracy is 0.5 to 1.0 mg., depending on the molecular weight.

RESULTS

The chyle fat during a low-fat diet

The chyle fat does not give an accurate impression of the intestinal lymph fat because the chyle consists of a mixture of intestinal and non-intestinal lymph. This should be kept in mind when a comparison is made of the fatty acid composition of chyle fat with that of dietary fat. In other words, if the chyle fats are to be compared with the corresponding dietary fats, a correction must be made for the admixture with non-intestinal lymph fat. The composition of the fat in the non-intestinal lymph is unknown, but it may be assumed that chyle fat collected during a low-fat period is similar to non-intestinal lymph fat, because the fat in the chyle during such a low-fat diet can not be of intestinal origin. The correction factor was determined by analyzing the chyle fat of four low-fat periods. Both the fatty acid composition and the fat content of this chyle were practically constant in all the periods. From this it follows that the composition of the "corrected chyle fat" can be calculated by subtracting the non-intestinal fat from the chyle fat as found by analysis. Then the fatty acid pattern of the "corrected chyle fat" can be compared directly with that of the dietary fat.

The patient was on a low-fat diet for four successive periods of a week each, the amounts of

[1] The protein milk was prepared by Nutricia, Ltd., of Zoetermeer, The Netherlands, and consisted of a base of buttermilk in which 5.5 gm. per 100 ml. butterfat, coconut oil, olive oil, etc. were emulsified without the addition of an emulsifier. In some periods the fat was emulsified in skimmed milk.

[2] One tablet of Vitamin B-complex daily was administered, containing: thiamin 5 mg., riboflavin 2 mg., nicotinic acid amide 20 mg., adermin 2 mg., and calcium pantothenate 3 mg.

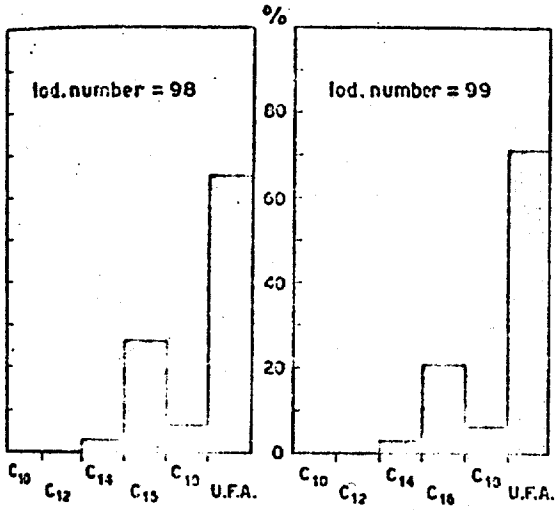

FIG. 1. LEFT: COMPOSITION OF THE PATIENT'S CHYLE FAT DURING A LOW-FAT DIET (LESS THAN 1 GM. FAT DAILY FOR FOUR WEEKS). RIGHT: COMPOSITION OF THE BLOOD FAT OF THE FASTING PATIENT (DURING A DIET CONTAINING RAPESEED OIL)

Solid columns = saturated fatty acids.
Striped columns = unsaturated fatty acids (U.F.A.)

fat given being less than 1 gm. daily. The chyle, which was of a milky white color during a regimen with normal quantities of fat (28 gm. daily), became transparent during the low-fat diet; in appearance it resembled blood plasma. The fat content of the chyle fell markedly, from 3.7 to about 0.6 gm. per cent.

Figure 1 shows the composition of the chyle fat during a low-fat regimen. For purposes of comparison the fatty acid pattern of the blood fat of the fasting patient during another dietary period is also given. There is a striking resemblance between the two fatty acid patterns (31). This does not imply that the forms in which these fatty acids occur (triglycerides, phospholipids, and cholesterol esters) are also similar. Table II shows that during the first period of the low-fat diet there was still an admixture with "high-fat" chyle from the preceding margarine period. In the three following periods, however, the chyle was characterized by a constant low fat content and a constant fatty acid composition. The iodine number of this chyle fat was as high as that of the fat of the blood serum of the fasting patient (see Figure 1).

Table II also shows the composition of the chyle fat of a patient with chyloascites from the Hospital for Sick Children, Great Ormond Street, London. The chyle of this nine-month-old patient was collected during a low-fat diet and was put at our disposal by Drs. W. W. Payne and A. Norman. It appears from these data that the fatty acid pattern of the chyle from their patient shows a resemblance to that of the "low-fat" chyle from ours (see Table II).

The chyle fat during a diet containing butterfat, coconut oil or margarine

The dietary fats and the chyle fats were analyzed in the usual manner, while the n-tetranoic acid content was determined separately by the method of Fairbairn and Harpur (32). The results are shown in Figure 2. The figures on the left show the percentage composition of the dietary fat in comparison with the "corrected" composition of the chyle fat. Those on the right give curves of the lymphatic absorption coefficient of each fatty acid of the dietary fat. These coefficients were cal-

TABLE II

The per cent of fatty acids in the fat from the chylothorax and chyloascites of our patient and the London patient, respectively

Fatty acid	Chylothorax patient					Chyloascites patient
	Margarine diet 28 gm. per day	Low-fat diet I	Low-fat diet II	Low-fat diet III	Low-fat diet IV	Low-fat diet
n-Decanoic (C_{10})	1	—	—	—	—	—
n-Dodecanoic (C_{12})	10	7	<1	1	0	1
n-Tetradecanoic (C_{14})	9	7	3	2	2	2
n-Hexadecanoic (C_{16})	19	21	26	26	28	25
n-Octadecanoic (C_{18})	6	6	6	6	7	10
n-Eicosanoic (C_{20})	—	—	—	—	—	1
Total unsaturated fatty acids	55	59	64	66	63	61
Iodine number	64	77	93	93	89	58
Total fat content of chyle, in per cent	3.70	1.22	0.54	0.52	0.58	0.21
Coefficient of fat absorption, in per cent	96	—	—	—	—	—

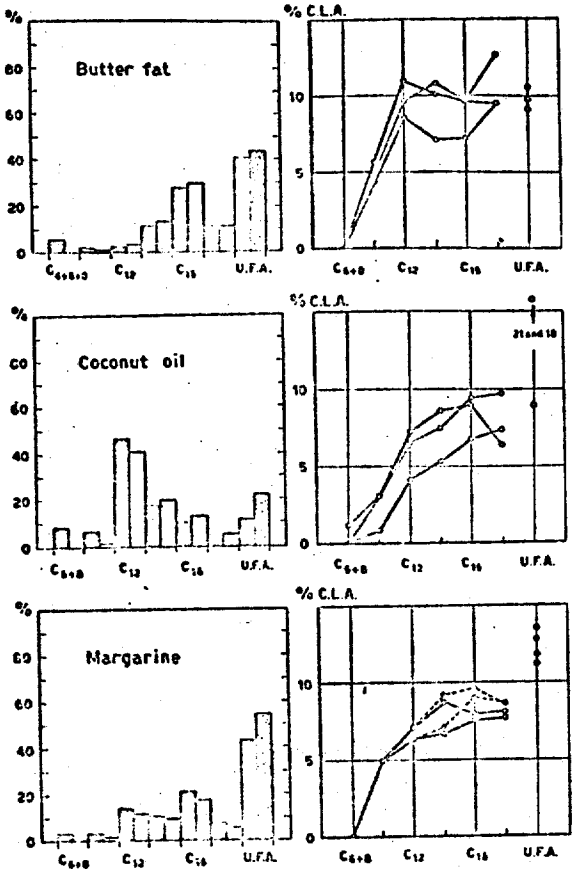

FIG. 2. LEFT: COMPOSITION OF DIETARY FAT AND OF CHYLE FAT (CORRECTED) DURING DIETS CONTAINING RESPECTIVELY BUTTER FAT, COCONUT OIL AND MARGARINE. RIGHT: COEFFICIENTS OF LYMPHATIC ABSORPTION (C.L.A.) OF THE SATURATED AND UNSATURATED FATTY ACIDS

Solid columns = saturated fatty acids in dietary fat.
Open columns = saturated fatty acids in chyle fat.
Striped columns = unsaturated fatty acids in dietary fat.
Stippled columns = unsaturated fatty acids in chyle fat.
O———O = "corrected values."
o - - - - - - - - o = "uncorrected values."

culated by determining the percentage of each fatty acid of the dietary fat which was recovered in the chyle. This percentage is called the "coefficient of lymphatic absorption" (C.L.A.) of the fatty acid. We are, of course, quite aware that these absorption coefficients do not indicate the total absorption of a given fatty acid via the lymph because the drainage of chyle from the patient was only partial. Only about 10 per cent of the fat from the food is recovered as chylé fat. This quantity, which was about 15 gm. chyle fat per diet period, is large enough for the calculation of the C.L.A. of each fatty acid as follows:

$$\text{C.L.A.} = \frac{\text{mg. fatty acid in chyle}}{\text{mg. fatty acid in food}} \times 100$$

These figures, therefore, merely indicate how much fatty acid of the dietary fat was found in the aspirated chyle, which was only part of the total chyle. The C.L.A.'s, however, permit comparison of the various fatty acids with each other with respect to their lymphatic absorption.

The diagrams demonstrate a resemblance between the composition of the chyle fat and the dietary fat. This holds true for butterfat, coconut oil, and margarine. On closer consideration, however, certain differences become clearly manifest when the various C.L.A.'s are taken into account (see the graph on the right). n-Tetranoic, n-hexanoic and n-octanoic acid are completely absent in the chyle; the C.L.A. of n-decanoic acid is 3 to 5 per cent; of n-dodecanoic acid 7 to 10 per cent; of n-tetradecanoic acid somewhat more, just as for n-hexadecanoic and n-octadecanoic acid. In this way it is demonstrated that the C.L.A. increases when the chain length of the fatty acids increases. The unsaturated fatty acids are absorbed as well as or somewhat better than the saturated n-tetradecanoic, n-hexadecanoic, and n-octadecanoic acids.

The chyle fat during a diet containing "caprylolive oil"

As it was a very striking feature that the short-chain fatty acids, n-tetranoic, n-hexanoic, and n-octanoic, were not found in the chyle when the patient was given margarine, butter, or coconut oil, it was of importance to verify this observation by administering one of these fatty acids in a greater amount in the form of triglyceride. The following data gathered from the literature are given for purposes of comparison. Kiyasu, Bloom, and Chaikoff (33) administered to rats 1-C^{14}-labelled n-decanoic acid dissolved in maize oil. The lymphatic absorption of this free fatty acid was only 7 to 19 per cent. Frazer used tributyrine (the triglyceride of n-tetranoic acid) which was not found in the chyle after administration to rats. One should, however, be cautious in drawing con-

clusions from these experiments because a free fatty acid was used in the first experiment, which is an unphysiological procedure. In the second experiment a single triglyceride was employed, not to be compared with the natural fats which are composed of mixed triglycerides. Moreover, in contrast to the other long chain fatty acids, n-tetranoic acid is very readily soluble in water. Apart from these objections the experiments show at most that some short-chain fatty acids are not absorbed in the chyle or absorbed only to a very slight degree. We observed the same phenomenon in the absorption of mixed triglycerides in which the short-chain fatty acids, n-tetranoic, n-hexanoic and n-octanoic acid, from margarine, butterfat, and coconut-oil were also absent in the chyle. At the same time, however, the long-chain fatty acids were demonstrated in the chyle. This can only be explained by hydrolysis of the dietary fat.

In the experiment described below, a fat was chosen which would clearly show whether or not hydrolysis occurred. Use was made of "capryl olive oil," a fat containing 46 per cent n-octanoic acid. This was prepared by subjecting a mixture of equal parts of olive oil and tricapryline, the triglyceride of n-octanoic acid, to alkaline re-esterification. In this way a mixed triglyceride was formed in which each molecule contained at least one n-octanoic acid group.[3] This "capryl olive oil" was given to the patient in the usual quantities (23 gm. daily) for one week. The fat balance was normal during this period (absorption coefficient 93 per cent). The fat content of the chyle also retained a normal value (2.1 per cent). This proves that the fat absorption in general, as well as the lymphatic absorption in particular, was normal. For these reasons this experiment with a synthetic fat may be considered a physiological one.

Figure 3 shows that the chyle fat contained only 4 per cent n-octanoic acid in contrast to the dietary fat which contained 46 per cent of this acid. The C.L.A. of n-octanoic acid is only 0.70 per cent, while that of Δ^9-octadecanoic acid is 8.6 per cent. These experiments therefore confirm the findings

[3] The composition of the "capryl olive oil" was calculated as: 27.5 mol. per cent tricapryline; 44.3 mol. per cent "dicapryl olive oil;" 23.9 mol. per cent "monocapryl olive oil;" and 4.3 mol. per cent olive oil.

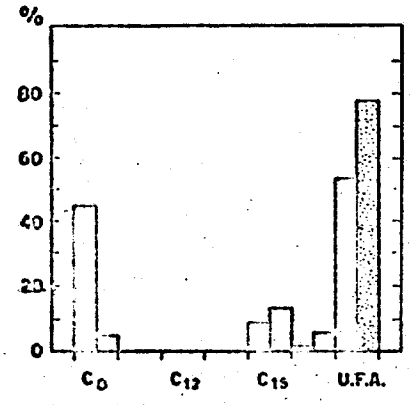

FIG. 3. COMPOSITION OF DIETARY FAT AND OF CHYLE FAT (CORRECTED) DURING A DIET CONTAINING "CAPRYL OLIVE OIL"

Solid columns = saturated fatty acids in dietary fat.
Open columns = saturated fatty acids in chyle fat.
Striped columns = unsaturated fatty acids in dietary fat.
Stippled columns = unsaturated fatty acids in chyle fat.

when butterfat, coconut oil, and margarine are used.

As n-octanoic acid was found in the chyle in only slight amounts, while the "capryl olive oil" was well absorbed (the fat balance was normal), it seemed acceptable that this fatty acid was absorbed via the portal vein or converted in the intestinal wall (< 12.5 mg. per cent octanoic acid). Whether n-octanoic acid could be demonstrated in the blood was therefore investigated. Three and one-half hours after an oral dose of 23 gm. "capryl olive oil" (*i.e.*, the whole amount for one day) venepuncture was carried out and the blood fat analyzed. n-Octanoic acid could not be demonstrated in it, and it may therefore be concluded that n-octanoic acid does not pass the liver as such but is converted in the intestine or liver.

The chyle fat during a diet containing cacao-butter and hydrogenated herring oil with peanut oil

In order to study in more detail the lymphatic absorption of the saturated fatty acids, n-hexadecanoic, n-octadecanoic, n-eicosanoic and n-docosanoic, experiments were carried out with cacao-butter, a fat containing 25 per cent n-hexadecanoic and 35 per cent n-octadecanoic acid, and with a hydrogenated herring oil (re-esterified with peanut oil so that the melting point was 42°C.), a fat containing 8 per cent n-eicosanoic and 8 per cent

n-docosanoic acid. No conclusions can be drawn regarding the absorption of n-hexadecanoic and n-octadecanoic acid because the cacao-butter diet was preceded by a period during which the patient suffered from enteritis. Although he seemed clinically cured, there was still present a steatorrhea (absorption coefficient = 80 per cent), the fat of which contained much hexadecanoic and n-octadecanoic acid. The low coefficient of absorption of cacao-butter might be caused by the high percentage of stearic acid in this fat, *viz.* 35 per cent. This is in accordance with the results of Bloom, Chaikoff, and Reinhardt (23) who found that only 36 to 54 per cent of stearic acid, as free fatty acid dissolved in corn oil, is absorbed by the rat, in contrast to at least 80 per cent of shorter chain fatty acids.

It can only be deduced that n-eicosanoic (C_{20}) and n-docosanoic (C_{22}) acids are absorbed through the lymph. It was, however, impossible to calculate the degree of this absorption because the chyle production had fallen markedly during this period, owing to the patient's spontaneous recovery.

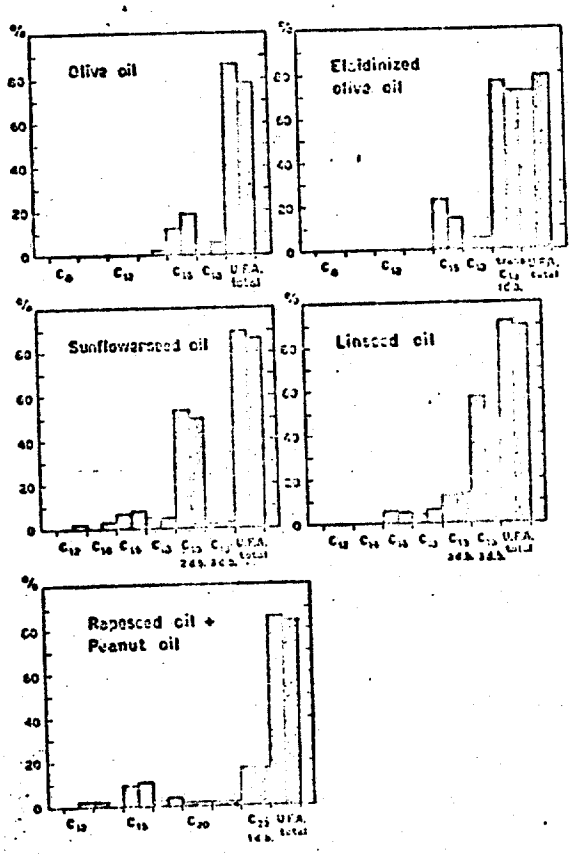

FIG. 4. COMPOSITION OF DIETARY FAT AND OF CHYLE FAT (CORRECTED) DURING DIETS CONTAINING FATS WITH UNSATURATED FATTY ACIDS

Solid columns = saturated fatty acids in dietary fat.
Open columns = saturated fatty acids in chyle fat.
Striped columns = unsaturated fatty acids in dietary fat.
Stippled columns = unsaturated fatty acids in chyle fat.

The chyle fat during diets containing fats with unsaturated fatty acids

In order to study more closely the absorption of various unsaturated fatty acids, Δ^9-octadecenoic acid (cis and trans-isomers), $\Delta^{9,12}$-octadecadienoic acid, $\Delta^{9,12,15}$-octadecatrienoic acid, and Δ^{13}-docosenoic acid, experiments were carried out with olive oil (containing cis-Δ^9-octadecenoic acid), elaidinized olive oil (containing trans-Δ^9-octadecenoic acid), sunflower seed oil (containing $\Delta^{9,12}$-octadecadienoic acid and $\Delta^{9,12,15}$-octadecatrienoic acid), linseed oil (*idem*) and rapeseed oil + peanut oil (containing Δ^{13}-docosenoic acid). The results are represented in Figure 4. In all cases there was a great degree of resemblance between the composition of chyle fat and dietary fat.

Summary

The results of the entire investigation are comprised in Figure 5. The composition of the chyle fat remained practically unchanged in successive periods when the same dietary fat was given. On the other hand, when a change was made in the dietary fat a simultaneous change in the chyle fat was observed. The similarities and differences between the dietary fat and the corresponding chyle fat are also evident in the Figure.

DISCUSSION

Experiments have indicated that the fatty acid pattern of the chyle fat differs from that of the dietary fat.

Of the *saturated fatty acids*, n-tetranoic and n-hexanoic are not present in the lymph, while n-octanoic is found only to a very slight degree, even if this fatty acid is present in large amounts in the dietary fat. n-Decanoic acid is found in the lymph but its absorption is less than that of n-dodecanoic, n-tetradecanoic, n-hexadecanoic, and

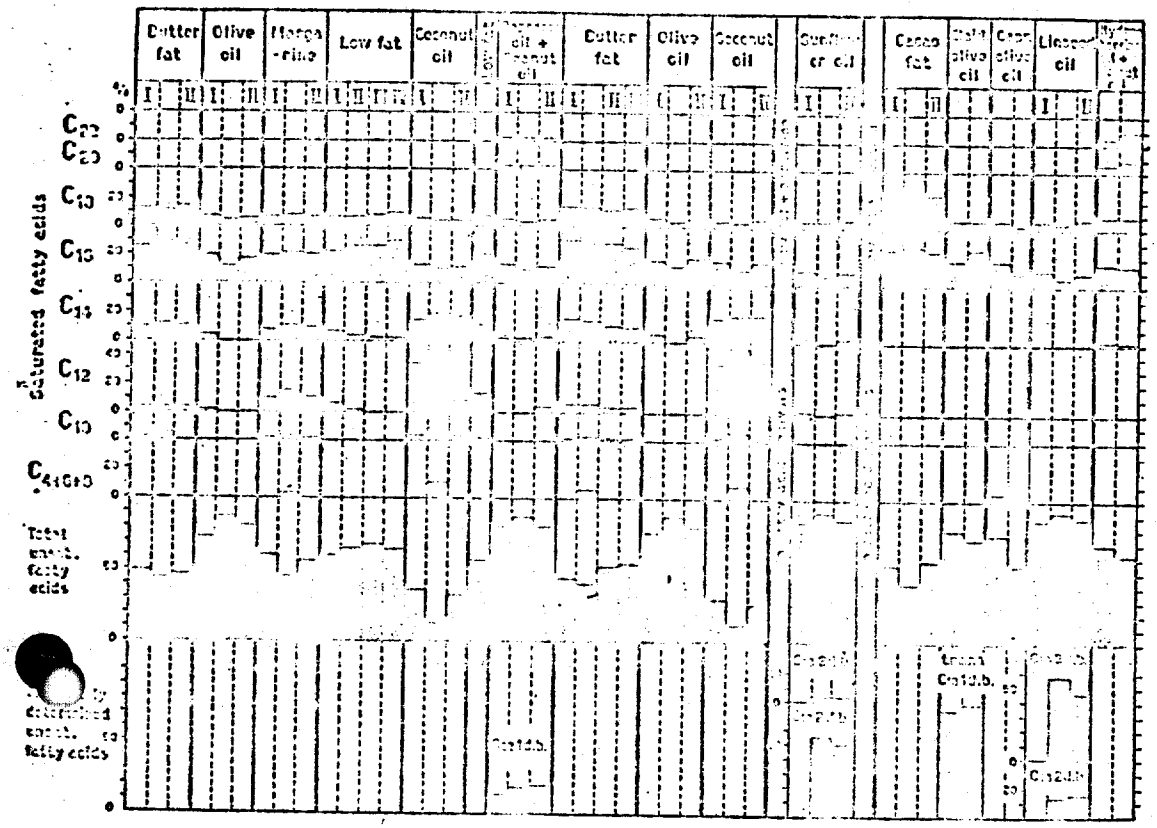

FIG. 5. COMPARISON OF THE FATTY ACIDS OF THE DIETARY FAT WITH THE FATTY ACIDS OF THE CHYLE, UNCORRECTED VALUES, REPRESENTED IN CHRONOLOGICAL ORDER DURING ALL DIETS OF THE WHOLE PERIOD OF OBSERVATION

Solid columns = fatty acids of diet.
Stippled columns = fatty acids of chyle.

n-octadecanoic. The four last-mentioned fatty acids are absorbed practically to the same extent. This can be deduced from Figure 6 which shows the coefficients of lymphatic absorption (C.L.A.) of the saturated fatty acids of various fats. The lines run closely parallel in the same direction which proves clearly that the C.L.A.'s of n-hexanoic, n-octanoic, n-decanoic, and n-dodecanoic acid increase as the carbon chains become longer.

In order to compare the C.L.A.'s of the fatty acids in the various periods, it is necessary to eliminate the differences in the production of chyle during these periods (see Figure 7). Although some objections may be raised to this procedure, the fatty acids found in the chyle were corrected for a standard chyle production of 500 cc. per week. This seems to be justifiable since the fat content of the chyle was independent of the quantity of chyle produced per week and also because the amount of chyle was independent of the nature of the dietary fat. The diminution of the quantities of chyle aspirated weekly was due to the patient's spontaneous recovery.

The various *unsaturated fatty acids* were absorbed well. The C.L.A.'s of these fatty acids are of the same order as those of the saturated fatty acids, n-tetradecanoic, n-hexadecanoic, and n-octadecanoic.

It follows from the difference in lymphatic absorption of the saturated fatty acids, n-octanoic, n-decanoic, and n-dodecanoic, that the dietary fat must be hydrolyzed at least partially during absorption. Otherwise, if dietary fat was absorbed without hydrolysis, there would be no difference in the absorption coefficient of the three mentioned fatty acids.

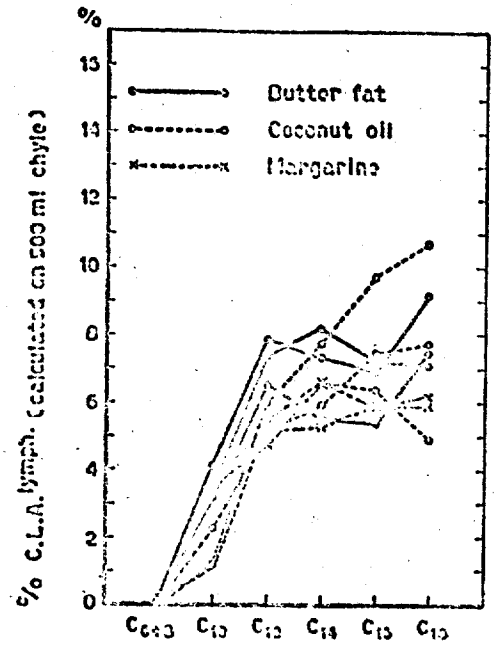

FIG. 6. COEFFICIENTS OF LYMPHATIC ABSORPTION (C.L.A.) OF VARIOUS DIETARY FATS CALCULATED FOR 500 ML. CHYLE

Thus the results of the experiments with butter, margarine, and coconut oil may be explained by hydrolysis of *part* of the dietary fat. The other part of it might pass through the intestinal wall as triglyceride and be absorbed with the chyle. In the experiment with "capryl olive oil" almost every molecule of fat must have been hydrolyzed, as Figure 5 shows that the C.L.A. of n-octanoic acid is only 0.7 per cent, while the coefficient for the longer chain fatty acids of this fat amounts to 8.6 per cent.

The result of the very low lymphatic absorption of n-octanoic acid is also clearly manifest on comparison of the composition of dietary fat. The dietary fat contained 46 per cent n-octanoic acid while only 4 per cent of this acid was recovered in the chyle fat. The practical absence of n-octanoic acid in the chyle fat can only be explained by the assumption of a hydrolytic breakdown of almost every molecule of fat because each fat molecule contains an n-octanoic acid group. This hydrolysis, however, is not conceivable when the fat is present in the intestine in the form of tiny globules as the lipase cannot act on each fat molecule under these circumstances.

On the strength of the above considerations ("capryl olive oil"), the assumption that practically every triglyceride molecule undergoes hydrolysis does not imply that each fat molecule is hydrolyzed completely (1–2); it is quite possible that only one or two molecules of fatty acid are split off, so that mono- and diglycerides are formed.

It may be that the intestine possesses the ability of dealing with a fat, molecule by molecule. Hence the following differentiation might be possible:

1. Certain fats might be absorbed almost without hydrolysis, *e.g.*, the greater part of the markedly unsaturated fats, olive oil, sunflower seed oil, linseed oil, and rapeseed oil.

2. Other fats must first be hydrolyzed completely or partially, *viz.* the fats containing short-chain fatty acids. After resynthesis, transport to the chyle follows, but the liberated short-chain

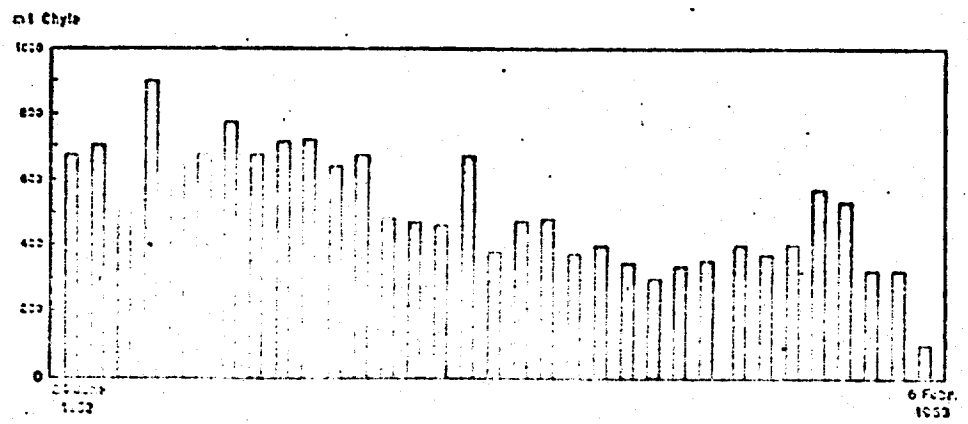

FIG. 7. THE QUANTITY OF CHYLE ASPIRATED DURING THE SUCCESSIVE PERIODS OF THE INVESTIGATION

fatty acids are converted in the liver or in the intestinal cells. This may be the case in the absorption of "capryl olive oil," butter, margarine, and coconut oil.

This hypothesis concerning the absorption of fats implies that the droplets of the fat emulsion in the intestine (3–12) disintegrate into molecules during absorption, with, if necessary, a selection of the fatty acids of which these molecules are built up. The fat droplets in the chyle are consequently of a different composition from the fat droplets of the dietary fat in the intestinal lumen, unless we are dealing with the absorption of the unsaturated fats.

A selective splitting off of special fatty acids from the *fat droplets* is hardly probable, the more so because these are surrounded by a layer of mono- and diglycerides (3–12). Frazer's view that the fatty acids n-tetranoic to n-decanoic are removed selectively from the fat droplets after hydrolysis, owing to their solubility in water, is debatable, because only the solubility of n-tetranoic acid in water is very high, while there of n-hexanoic, n-octanoic and n-decanoic acid decrease successively with rapidity. The above selection may take place in the intestinal contents or in the intestinal cells.

CONCLUSIONS

It may be stated that the nature of a dietary fat decides whether or not it is hydrolyzed in the intestinal lumen and subsequently partially resynthesized to chyle fat in the intestinal wall. If there is a hydrolysis followed by resynthesis, these processes are such that the degree of lymphatic absorption is different for each fatty acid. This means that the absorption mechanism is accompanied by considerable selective capacity. Selection of triglycerides or fatty acids, however, is only possible if the fat droplets, each actually consisting of millions of molecules, disintegrate.

On the strength of this idea it is conceivable that some dietary fats (olive oil, sunflower seed oil, etc.) pass more or less unchanged into the chyle, and that in the case of other fats (butter, coconut oil, margarine, etc.) the fatty acid pattern of the chyle fat differs considerably from that of the dietary fat.

SUMMARY

The absorption of fat via the lymph was studied in an otherwise healthy child with chylothorax by administering various dietary fats during two-week periods and comparing the fatty acid composition of dietary fat and chyle fat.

It appeared that the various fatty acids were not absorbed to the same extent. n-Hexanoic, n-octanoic and n-decanoic acid were not found, or found to only a slight degree, in the chyle. This was confirmed with an especially prepared fat, "capryl olive oil," containing 46 per cent n-octanoic acid.

The following hypothesis was evolved, based on the experiments described: Certain fats may be absorbed almost without hydrolysis, *e.g.*, the greater part of the markedly unsaturated fatty acids (such as olive oil, sunflower seed oil, and rapeseed oil). Other fats must first be hydrolyzed completely or partially, *viz.*, the fats containing short-chain fatty acids, such as butter, coconut oil, and "capryl olive oil." In the latter case this hypothesis cannot be reconciled with Frazer's theory of particulate absorption.

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clinical responses varied considerably in severity. The difference strongly suggests that the amount of effective virus in the inoculum had a significant influence upon the result. If this should not be the case one might suggest that a variation in the virus had occurred during its 2 passages by parenteral inoculation in human subjects. In any case the data illustrate the difficulty in classifying infectious hepatitis on the basis of the incubation period after parenteral injection of icterogenic serum. The relative uniformity in the time of onset of jaundice in the inoculated individuals indicates, on the other hand, that the variation in severity of the clinical courses following the same inoculum is related to individual susceptibility.

The events demonstrate how, with virus in the blood well before the onset of symptoms, infectious hepatitis may be transmitted by blood products derived from individuals in whom the presence of the virus would be entirely unsuspected.

Summary. A transfusion of blood from an individual in the incipient stage of infectious hepatitis elicite ms in the recipient after 1 ice after 21 days.

By subcutaneous in 45 ion volunteers the virus in the serum of the recipie day after transfusion which was 5 days before the first symptoms and 13 days before the onset of jaundice. Virus was also demonstrated in another specimen of serum taken 6 days later, or 3 days after first symptoms.

The incubation period as measured by the onset of jaundice in the 4 subjects who had definite illness was 42 to 47 days, while the clinical response varied greatly in severity.

The significance of the results is discussed.

This investigation was made possible by the cooperation of Dr. Garrett Heyns, Director of Corrections, State of Michigan, and Dr. W. B. Huntley, Medical Director of the State Prison of Southern Michigan, Jackson, Michigan, and his staff, to whom the authors wish to express their appreciation for constant assistance. They wish also to express their indebtedness to the volunteers who participated so willingly and cooperatively in the study.

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Sources of Energy for Intestinal Smooth Muscle Contraction.*†

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The rhythmic contractions of a segment of rabbit small intestine, suspended in glucose-free Tyrode solution, were found by Rona and Neukirch[1,2,3] to undergo a gradual and progressive decrease in amplitude; at which time, the addition of certain substances (glucose, mannose, pyruvic, lactic, acetic, butyric, β-hydroxybutyric and oxaloacetic acids) served to increase and maintain the amplitude of contraction, the extent of the increase varying with the substance added. They attributed the progressive decrease in the height of the contractions in the absence of glucose to gradual depletion of the endogenous stores

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† We are indebted to Dr. David Rittenberg for samples of enanthic and pelargonic acids, and to Dr. Severo Ochoa for the α-ketoglutaric acid used in this study.

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of energy-yielding substrates. The effectiveness of a substance in restoring amplitude was considered a measure of its ability to serve as a source of energy for smooth muscle contraction. More recently, Feldberg and Solandt,[4] and Feldberg,[5] in extending the work of the earlier investigators, have arrived at the same conclusions.

Except for the studies cited, the procedure introduced by Rona and Neukirch has not been utilized for the investigation of the intermediary metabolism of smooth muscle. If their assumption is correct, that the restoration of amplitude is evidence of the ability of a substance to provide energy for smooth muscle contraction, then the method would appear to be unusually well suited for such studies. The restoration of contractility would comprise a more exacting and reliable criterion for the evaluation of a substrate, than is provided by a rise in oxygen consumption alone. The latter criterion is frequently undependable; the former furnishes direct evidence that the energy provided by a substance under study is actually utilizable for smooth muscle contraction.

In the present study the method has been employed for a more extensive investigation of potential energy-yielding substrates, selected on the basis of modern concepts of intermediary metabolism, and particular attention has been given to the possible usefulness of fatty acids.

Methods. The standard preparation consisted of a fresh segment, approximately 4 cm long, from the upper portion of rabbit small intestine. The segment, with its lumen open, was suspended in a 50 cc muscle chamber, and attached to an isotonic muscle lever. The lever exerted a tension of 4 g, and recorded the contractions of the longitudinal muscle on a kymograph. The intestinal segment was bathed in glucose-free Krebs-Henseleit solution,[6] maintained at 37.5°, and aerated with 95% O_2 - 5% CO_2 to obtain a pH of 7.4.

Prior to testing each substance, the spontaneous rhythmic contractions of the longitudinal smooth muscle were allowed to decrease to about 1/10 to 1/20 of the original amplitude. This usually required less than 1 hour and introduced no change in contraction frequency. At this point, the test substance, dissolved in a small quantity of Krebs solution, was added to the muscle chamber. All acids were used in the form of sodium salts. For each substance tested, the minimal concentration was determined which brought about the maximal amplitude of contraction of which that substance was capable. This increase in amplitude was then compared on a percentage basis with the maximal change which resulted from the addition of glucose to the same intestinal preparation.

Results. The record of a typical experiment is reproduced in Fig. 1. It illustrates the gradual decrease in amplitude and average tonus which takes place in the absence of substrate as well as the restoration of both on the addition of glucose (200 mg %) when the amplitude had fallen to about 1/10 to 1/20 of the original value. The amplitude and average tonus began to increase within a minute of the addition of glucose, and the maximal amplitude was reached within 10 minutes, and maintained thereafter. In this experiment, the maximal amplitude following glucose was greater than that initially exhibited; in other experiments, the amplitude attained after the addition of glucose was either equal to, or greater than, but never less than, the original amplitude. Not discernible in the tracing because of the reduction in size, are the number of contractions per minute given by the intestinal segment and the constancy of this frequency throughout the experiment, despite marked changes in amplitude. Under our experimental conditions, the contractions averaged 17 per minute with most of the intestinal segments used. This uniformity of contraction rate throughout the experiment simplified the comparison of the relative effectiveness of different substrates in providing energy for contraction, since the only variable introduced consisted of a change in amplitude.

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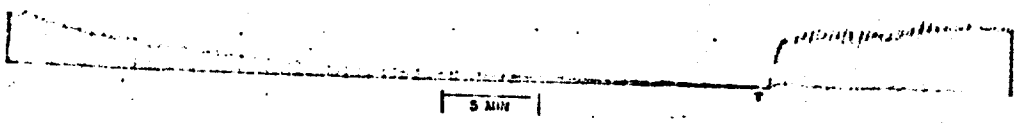

Fig. 1.
Kymograph tracing showing decrease in amplitude of intestinal contractions in glucose-free solution and restoration of contraction amplitude on addition of glucose (at arrow) to provide a concentration of 200 mg %.

The second step in the experimental procedure—that is, the comparison of the relative effectiveness of different substrates in supporting smooth muscle contractions—is illustrated in Fig. 2. The results obtained with 4 substrates are expressed in semi-diagrammatic fashion, each contraction wave representing approximately 17 contraction waves in the actual kymograph tracing. With the amplitude after addition of glucose as the standard of reference, the relative effectiveness of the other substrates is as follows: pyruvic acid, 100%; butyric acid, 50%; and enanthic acid, 20%.

In Tables I and II, are assembled the results obtained with a number of substances which proved capable of restoring, in varying degrees, the amplitude of smooth muscle contraction. In Table I, the height of the response is compared with that produced by glucose, and, for each substance, represents the maximal response which it can elicit. Table II lists the minimal concentration of each substrate required to bring about its individual maximal increase in amplitude.

Table III lists those substances which failed

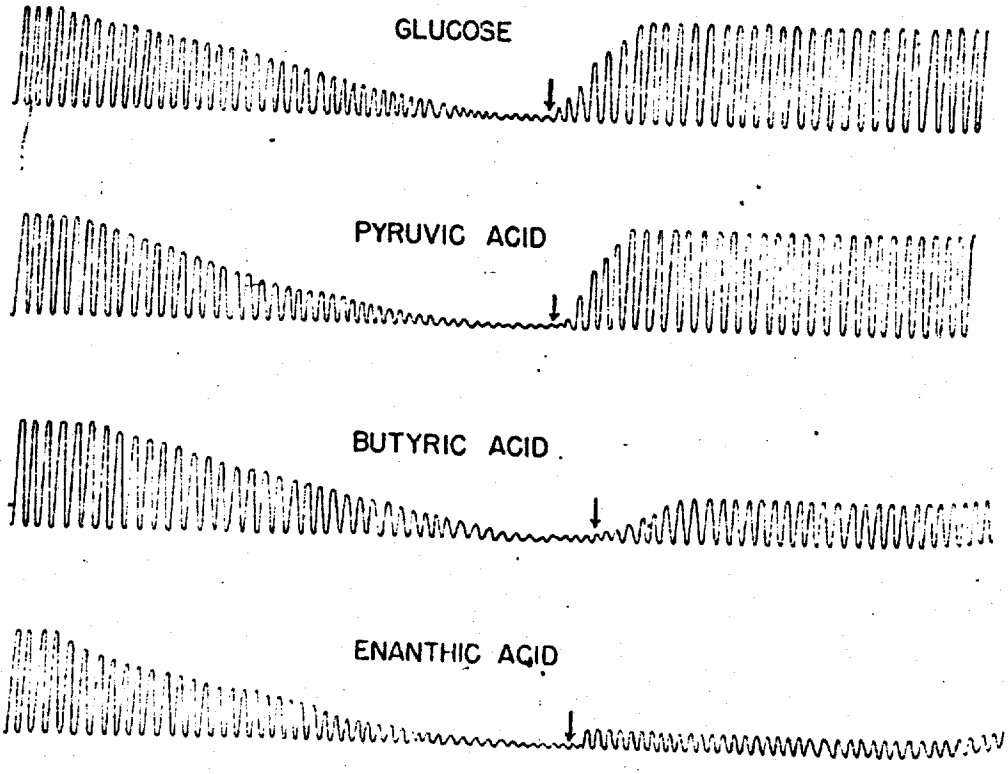

Fig. 2.
Semi diagrammatic representation, based on kymograph tracings, illustrating relative effectiveness of glucose, pyruvic, butyric, and enanthic acids in restoring amplitude of contraction of intestinal smooth muscle after prior exposure to a glucose-free solution.

TABLE I.
Substances which Increase Amplitude of Contraction.

Substance	Response compared with that of glucose
	%
Glucose	100
Mannose	70-90
Acetic acid	100
Pyruvic acid	100-110
Oxaloacetic acid	75-100
DL-Lactic acid	20-40
Acetoacetic acid	20-30
Butyric acid	40-60
Caproic acid	40-60
Caprylic acid	40-60
Pelargonic acid	35-45
Enanthic acid	15-20
Valeric acid	5-10

to increase the amplitude of contraction. Each was tested over a range of concentration extending from 10 to 100 mg %, and found to be without influence on the gradual reduction in tone and amplitude of contraction characteristic of the preparation in the absence of substrate. There were 2 exceptions: acetaldehyde and glyceraldehyde, in concentrations of 100 mg %, exerted a marked inhibitory effect, as evidenced by the rapid and virtually complete cessation of all contractile activity. The inhibition induced by acetaldehyde could not be overcome by the subsequent addition of glucose, pyruvate, acetate or lactate, whereas that caused by glyceraldehyde could be overcome by pyruvate, acetate, or any of the fatty acids listed in Table I, but not by glucose or lactate. In the case of citric acid, which removes Ca ions from solution to form a weakly dissociable

TABLE II.
Minimal Concentrations Necessary for Maximal Increases in Amplitude of Contraction.

Substance	Minimal concentration in mg %
Glucose	100-125
Mannose	125-150
DL-Lactic acid	20-30
Oxaloacetic acid	20-30
Acetic acid	10-15
Pyruvic acid	10-15
Butyric acid	4-5
Valeric acid	3-4
Caproic acid	2-3
Enanthic acid	1-2
Caprylic acid	0.5-0.75
Pelargonic acid	0.5

TABLE III.
Substances which Fail to Increase Amplitude of Contraction.

Galactose	Propionic acid
Fructose	Hydroxyacetic acid
Glycogen	Citric acid
Glucose-1-phosphate	Succinic acid
β-Phosphoglyceric acid	Fumaric acid
Glycerol	α-Ketoglutaric acid
dl-Glyceraldehyde	Glycine
Acetaldehyde	1(+)-Alanine
Ethanol	1(—)-Aspartic acid
Acetone	1(+)-Glutamic acid

(Ca citrate)$^{--}$ complex, the precaution was taken to add sufficient Ca ions (as $CaCl_2$) to replace those removed, in order to avoid changes in muscle tonus which would otherwise have resulted from a deficiency of Ca ions in the medium.

Fig. 3 is a graphic presentation of the maximal increases in amplitude observed with the series of fatty acids from acetic through pelargonic. The greatest increase, which equalled that given by glucose, was obtained with acetic acid. The remaining even-carbon fatty acids (butyric, caproic and caprylic) all produced an identical increase in amplitude, which was approximately 50% of that of acetic acid. On the other hand, the effects of the odd-carbon series of fatty acids were strikingly different. Propionic acid was devoid of effect. The changes in amplitude induced by the other members of the odd-carbon series were less (valeric, enanthic and pelargonic) than those obtained with even-carbon fatty acids, and differed in extent with each compound. The magnitude of response increased as the chain-length increased. Thus, the approximate increase with valeric acid was 10%, with enanthic acid 20%, and with pelargonic acid 40% of that observed with acetic acid.

Experiments of a preliminary character have been carried out to investigate the mechanisms responsible for the differences exhibited by odd- and even-carbon fatty acids. If β-oxidation takes place in smooth muscle, as our experiments suggest, the smaller fragments resulting from this type of degradation of the even-carbon fatty acids would all be utilizable for muscle contraction; whereas the odd-carbon fatty acids would leave a non-utilizable propionic acid residue. Since the

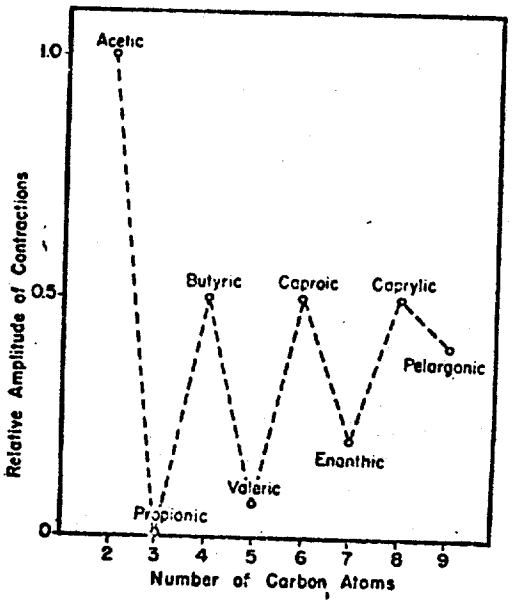

Fig. 3.
Relative effectiveness of odd- and even-carbon fatty acids in restoring amplitude of contraction of intestinal smooth muscle following prior exposure to glucose-free solution.

propionic residue would be formed at the surface of the postulated β-oxidation enzyme, it might conceivably compete for this surface with the unaltered fatty acid or its oxidizable fragments. This concept was explored by adding propionic acid in concentrations of 10-30 mg % to muscle preparations contracting under the influence of butyric or caprylic acid, in concentrations of 5.0 and 0.75 mg % respectively. In each instance, 30 mg % of propionic acid served to reduce the amplitude of contraction by about 80%. This inhibition, however, could subsequently be overcome by doubling the concentrations of butyric or caprylic acid.

The observation was also made that relatively high concentrations of enanthic, caprylic and pelargonic acids completely inhibited smooth muscle contraction. The effective concentrations varied with different intestinal preparations and were as follows: enanthic 50-200 mg %; caprylic 25-150 mg %; and pelargonic 20 mg % (1 experiment). This inhibition was not overcome by the addition of glucose, pyruvic acid, or acetic acid. However, it proved to be a reversible inhibition, since contractility was restored when the fatty acid was washed out and replaced with fresh Krebs solution.

Discussion. 1. *Mode of Action of Substances Which Restore Contractility.* Those substances which restore contraction amplitude may act either as substrates or pharmacological agents. All the evidence so far accumulated, although indirect, appears to favor the substrate concept. Rona and Neukirch[1] observed a reduction in glucose and mannose content during a 2½-hour period of active contractions. More specific evidence against a pharmacological action was provided by Feldberg and Solandt.[4] The addition of acetylcholine during a period of diminished activity in a glucose-free Tyrode solution produced a relatively small increase in tonus, sustained for less than a minute. However, following the restoration of amplitude by glucose or pyruvate, acetylcholine produced a marked and well-sustained increase in tonus. From this it was inferred that glucose and pyruvate do not increase smooth muscle activity by increasing the production of acetylcholine, and that this agent could exert its pharmacological effect only when energy for contraction was provided by appropriate substrates. These experiments have been confirmed and extended in our laboratory, with the observation that large and sustained increases in tonus may also be obtained with acetylcholine after the restoration of amplitude by fatty acids, such as acetate and butyrate. Although this evidence is indirect, we favor the substrate concept over the pharmacological, particularly in view of the conformity of much of the data obtained with prevailing views of intermediary metabolism. The discussion of our results has therefore been based on the concept that the capacity of a substance to augment and sustain contraction amplitude is derived from its ability to provide energy for smooth muscle contraction.

2. *Carbohydrate Series.* Of the hexoses studied, only glucose and mannose yielded energy for contraction; galactose and fructose were ineffective. In 2 respects our findings differ from those of Feldberg and Solandt.[4] We failed to find the increase in reactivity to acetylcholine reported by these investiga-

tors to follow the addition of fructose to a glucose-free medium. We also found mannose to be 70-90% as effective as glucose, in contrast with the 25-30% effectiveness which they observed. This latter discrepancy may be due to the fact that they did not use maximal increases in amplitude as the basis for comparison.

The excellent substrate effect of pyruvic acid is in accord with the Meyerhof cycle. The smaller effect produced by lactate is of interest. The lack of effectiveness of glycogen, glucose-1-phosphate, and β-phosphoglyceric acid need not be considered evidence against the occurrence of the Meyerhof cycle in smooth muscle since it is questionable whether the muscle cells are permeable to these substances. The same reservation holds for the ineffectiveness of glucose-6-phosphate and fructose-1,6-diphosphate, reported by Feldberg.[5]

3. *Acetic Acid.* This proved to be an excellent substrate, producing contraction amplitudes equal to glucose and pyruvate. This is compatible with recent observations on the utilization of acetate by a variety of tissues. The pathway for the oxidation of acetic acid in smooth muscle remains obscure; it does not appear to be oxidized to hydroxyacetic acid, since the latter had no substrate effect.

4. *The Krebs Tricarboxylic Acid Cycle.* Of the acids postulated in this cycle the following were tested and found ineffective: citric, α-ketoglutaric, succinic and fumaric. Oxaloacetic acid exerted a good substrate effect. However, the exceptional behaviour of oxaloacetic acid prompts the suggestion that it may have exerted its effect only after decarboxylation to the readily oxidizable pyruvic acid. The ineffectiveness of succinic acid is in accord with *in vitro* metabolic experiments with strips of dog intestinal smooth muscle (unpublished data). In these experiments carried out at reduced O_2 tensions, the increased O_2 uptake in the presence of succinic acid failed to increase the concentrations of phosphocreatine and adenylpyrophosphate, the high energy phosphates essential for muscle contraction. These results provide no support for the participation of the postulated tricarboxylic acid cycle in the activity metabolism of smooth muscle, and, by the same token, would exclude the cycle as a pathway for the oxidation of pyruvic and acetic acids.

5. *Amino Acids.* In conformity with the earlier observations of Neukirch and Rona,[2] the amino acids studied were found to exert no substrate activity. This was of particular interest with respect to alanine and aspartic acid, since their corresponding keto-acids, pyruvic and oxaloacetic, are extremely effective. Apparently, intestinal smooth muscle is unable to deaminate these amino acids to their keto forms.

6. *Ketone Bodies.* Neukirch and Rona[2] had previously reported a small increase in amplitude with β-hydroxybutyric acid. We have observed an effect of similar magnitude with acetoacetic acid, but none with acetone. Should the lack of utilization of acetone prove to be a general phenomenon, it might provide an explanation for the readiness with which acetone appears in the urine even with mild disturbances in fat metabolism.

7. *Fatty Acids.* The difference in the effects observed with odd- and even-carbon fatty acids strongly suggests that β-oxidation takes place in intestinal smooth muscle. Thus, butyric, caproic and caprylic, which can all be completely degraded to 2-carbon fragments, are all equally effective as substrates. On the other hand, propionic acid, which is not susceptible of β-oxidation, is ineffective; and the remaining odd-carbon fatty acids studied, all of which would leave a propionic acid residue on β-oxidation, have a smaller effect than those of the even-carbon series.

An explanation for the identical effects of the even-carbon fatty acids may be that they all undergo β-oxidation at the same enzyme surface; and that, once this surface is saturated with any one of them, β-oxidation then proceeds at the same rate. Support for this hypothesis is provided by the fact that once a maximal increase in amplitude is induced by any one of the series, the subsequent addition of another results in no further increase.

Our experimental results also permit a tentative explanation for the smaller effects of the odd-carbon acids (valeric, enanthic, pelargonic) as well as for the increase in

amplitude with the increase in chain-length. The β-oxidation of odd-carbon fatty acids should in every instance leave a non-utilizable propionic acid residue. Propionic acid has been found to reduce the effectiveness of even-carbon fatty acids as substrates, an inhibition which can be overcome by raising the concentration of the utilizable fatty acid. This suggests the competition of propionic acid for the surface of the β-oxidation enzyme. With the odd-carbon series, the competition between the propionic residue and the unaltered acid and its β-oxidizable fragments, would explain the smaller effects of this series. This competition should become less effective as the chain-length increased, since the β-oxidizable portion would progressively increase relative to the propionic acid residue.

One other factor may also contribute in part to the greater effectiveness of the odd-carbon fatty acids of longer chain-length. It was observed, for the whole series of fatty acids, that, as the chain-length increased, progressively smaller concentrations were required to produce maximal amplitude effects. An explanation for this finding may reside in the greater affinity of the longer-chain fatty acids for the β-oxidation enzyme surface, a concept which derives support from the increase in the adsorption coefficients of fatty acids at surfaces, as chain-lengths increase. With respect to the odd-carbon series, such an affinity relationship would [illegible] to the advantage of the longer-chain [illegible] in their competition with propionic residue.

No information is at present available as to the usefulness of higher fatty acids, such as oleic, palmitic, and stearic, as substrates for smooth muscle contractility, because of the extremely low solubility of their sodium salts in Krebs solution. They were ineffective in dilute suspensions.‡

Summary. A number of substances have been studied with respect to their influence on the contraction of excised segments of rabbit small intestine. The results have been discussed in relation to present concepts of intermediary metabolism. Particular attention has been given to fatty acids, from propionic through pelargonic. With the exception of propionic, all appear to provide energy for smooth muscle contraction; and the characteristics of their behaviour were compatible with the concept of β-oxidation.

‡ Since this manuscript was submitted, oleic acid has been found to partially restore contraction amplitude. It was introduced as a sodium oleate sol (100 mg %) in water along with enough NaCl to maintain isotonicity. The effective concentration of oleic acid could not be determined because of its partial precipitation.

We wish to acknowledge the technical assistance of Mathilda Fischl, Alice Kantor, and Rita Baff[illegible]

15301

Experimental Sinoauricular Block.*

DAVID SCHERF.

From the Medical Department, New York Medical College.

Sinoauricular block is a rare disturbance either of stimulus formation in the sinus node or of transmission of the stimulus to the auricle. In analogy with tracings obtained in [illegible]oventricular conduction disturbances, [illegible]ricular block is diagnosed whenever, with a regular sinus mechanism, pauses occur without evidence of auricular activity. These pauses are more or less approximate multiples of a normal period.

A disturbance of stimulus [illegible]ation in the sinus node or of stimulus [illegible]ion in the sinoauricular junctional fibers is understan[illegible]

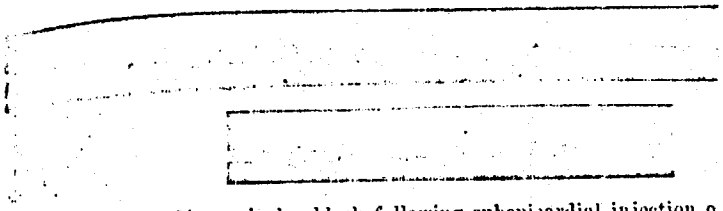

Fig. 1a and b. Sinoauricular block following subepicardial injection o[illegible] of the sinus node.

in patients with arteriosclerosis of the sinus node arteries is also to be expected. It is difficult to explain, however, the sinoauricular block which follows the administration of digitalis or the form which is occasionally seen in patients with myocardial involvement. The sinus node represents a relatively large structure with a length of more than 25 mm, it is united with the common auricular fibers by innumerable junctional fibers which spread in all directions. Furthermore, one must assume that all specific fibers of the sinus node are able to form automatic stimuli. It is hard to conceive how any but a very extensive process could lead to a sinoauricular block: the simultaneous periodical block of the sinoauricular conduction in all junctional fibers due to digitalis action seems impossible.

Therefore for many years Lewis spoke only of "sinoauricular block, so-called"[1] and Clerc discusses "le blocage sinoauriculaire ou supposé tel."[2]

In complete accord with these facts was the experience that in order to obtain sinoauricular block experimentally, it is necessary to isolate the sinus node on all 4 sides[3] to clamp the sinus node artery and thus damage the tissue of the whole node,[4] or to inject pituitary extracts which cause coronary spasm.[5] All experimentators who have tried to produce a sinoauricular block agree that this disturbance cannot be easily created.

In experiments which were performed for other purposes we registered electrocardio-

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[1] Lewis, T., *The mechanism and graphic registrations of the heart beat*, London, 1925.

[2] Clerc, A., *Les arythmies en clinique*, Paris, 1925.

[3] Cohn, A. E., Kessel, L., and Mason, H. H., [illegible] 1911-12, 3, 311.

[4] [illegible]

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Verhandl. Deut. Ges. Inn. Med. 74 (1968)

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anderen Patienten mit einer oberen Einflußstauung und röntg
dacht auf einen Mediastinalprozeß hatten wir im Bereich des
und Verbände atypischer Zellen festgestellt. In durch Brone
führter Probeexcision wurde ein Bronchialplattenepithelcarcinc
Auf Grund der eigenen 2jährigen Erfahrung sind wir der
Aspirationsbiopsie der Schilddrüse ihren praktischen Wert hat
logische Befund unter den übrigen Ergebnissen im Falle einer S
sollte.

D. GLAUBITT und H. FRAHM (I. Med. Univ.-Klinik und II. Med. Univ.-Klinik und Poliklinik, Hamburg-Eppendorf): Die $^{14}CO_2$-Exhalation bei Kranken mit substituiertem primärem Myxödem nach oraler Verabreichung von ^{14}C-Caprylsäure oder ^{14}C-Trioctanoat

Die $^{14}CO_2$-Ausatmung nach oraler Gabe einer ^{14}C-Fettsäure oder ihres ^{14}C-Triglycerids unterliegt dem Einfluß zahlreicher variabler Größen. Die kontinuierliche Messung der $^{14}CO_2$-Exhalation (s. auch VON HOLT u. SCHMIDT, 1961; TOLBERT u. COZZETTO, 1964) gestattet jedoch bei Berücksichtigung der den Ergebnissen zugrunde liegenden physiologisch-chemischen und biophysikalischen Vorgänge einen Einblick in den Intermediärstoffwechsel und erlaubt Hinweise auf die Fettverdauung und -resorption.

Bei Kranken mit substituiertem primären Myxödem hatten Umsatzuntersuchungen mit ^{131}J-Humanalbumin Abweichungen von den Normalwerten ergeben (GLAUBITT u. FRAHM, 1967), die für Störungen des Eiweißstoffwechsels sprachen. Um Anhaltspunkte für eine Beeinträchtigung auch des Fett- oder Intermediärstoffwechsels zu gewinnen, maßen wir bei diesen Kranken nunmehr die $^{14}CO_2$-Ausatmung nach oraler Verabreichung der gesättigten Fettsäure ^{14}C-Caprylsäure, die eine mittlere Kettenlänge besitzt, und später des entsprechenden Triglycerids ^{14}C-Trioctanoat.

Methodik

Wir untersuchten 5 gesunde männliche Probanden im Alter von 23 bis 26 Jahren, 2 Patienten im Alter von 31 und 34 Jahren und 4 Patientinnen im Alter von 21 bis 49 Jahren. Die Kranken standen unter einer Substitution mit täglich oral 0,10 oder 0,15 g Thyreoidea sicca. Zur $^{14}CO_2$-Exhalationsmessung erhielten sämtliche Personen nüchtern oral eine Suspension von 250 ml Vollmilch und 20 ml Maiskeimöl mit 5 µCi Caprylsäure-(1-^{14}C) und mindestens 1 Woche später 5 µCi Trioctanoat-(carbonyl-^{14}C) (NEN-Chemicals GmbH, Dreieichenhain; Buchler & Co., Braunschweig). Die spezifische Radioaktivität der ^{14}C-Caprylsäure betrug 1,99 mCi ^{14}C/mM., die des ^{14}C-Trioctanoats 7,55 mCi ^{14}C/mM.

Mit dem $^{14}CO_2$-Exhalationsmeßgerät FHT 50 A in weiterentwickelter Ausführung (Frieseke & Hoepfner GmbH, Erlangen-Bruck) registrierten wir die Konzentration des $^{14}CO_2$ und des gesamten Kohlendioxyds sowie die spezifische Radioaktivität in der Ausatmungsluft der Probanden. Die Untersuchungen dauerten bis zu 9 Std. [Weitere Einzelheiten der Methodik s. GEBAUER u. SUTTOR, 1966; GLAUBITT, 1967, 1968; GLAUBITT u. FRAHM, 1968 (1) und (2)]. Die Ergebnisse werden in Form des stündlichen Wertes der spezifischen Radioaktivität (nCi $^{14}CO_2$/M CO_2) angegeben. — Die Fettmenge im Stuhl an drei aufeinanderfolgenden Tagen wurde nach VAN DE KAMER u. Mitarb. (1949) bestimmt.

Ergebnisse

Die zweimalige Untersuchung der spezifischen Radioaktivität in der Ausatmungsluft bei gesunden Probanden und Kranken mit substituiertem primären Myxödem ergibt eine gute Reproduzierbarkeit der Resultate. Die Ausatmung des radioaktiv markierten Kohlendioxyds erfolgt bei manchen Kranken nach Erreichen des Maximums verzögert (Abb. 1). Da die Exhalation des gesamten Kohlendioxyds bei den Kranken im Normalbereich oder nur wenig niedriger liegt,

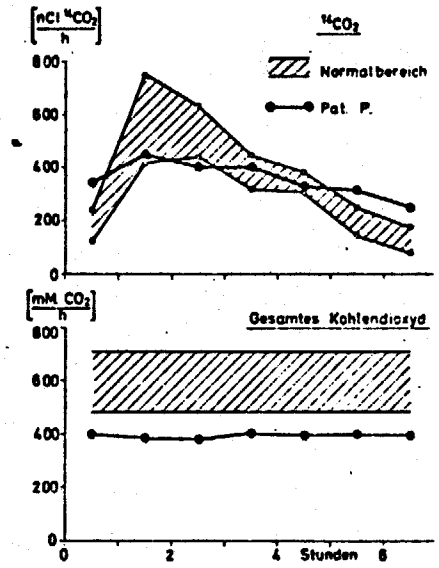

Abb. 1. Die durchschnittliche stündliche Konzentration des $^{14}CO_2$ und des gesamten CO_2 in der Ausatmungsluft einer Patientin mit substituiertem primären Myxödem nach oraler Gabe von ^{14}C-Caprylsäure

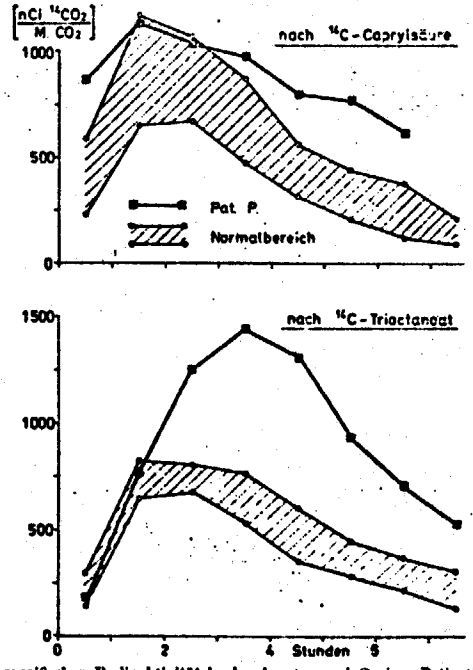

Abb. 2. Die Kinetik der spezifischen Radioaktivität in der Ausatmungsluft einer Patientin mit substituiertem primären Myxödem. (Durchschnittliche stündliche Werte; der Normalbereich ist gegeben durch die Extremwerte von insgesamt 8 Meßreihen bei 5 Gesunden)

Cap. Acid

ist die spezifische Radioaktivität in der Ausatmungsluft bei allen Kranken erhöht; dieser Befund ist meist mehrere Stunden nach Beginn der $^{14}CO_2$-Exhalationsmessung zu beobachten. Bei einer Patientin steigt die spezifische Radioaktivität in der 1. Std nach oraler Verabfolgung von ^{14}C-Caprylsäure wie auch von ^{14}C-Trioctanoat nicht so stark an wie bei den gesunden Probanden. Bei allen Kranken liegt sie nach Erreichen des Gipfels mehrere Stunden lang oberhalb der Normalwerte (Abb. 2).

Diskussion

Die eigenen Ergebnisse zeigen, daß bei den Kranken mit substituiertem primären Myxödem nach oraler Gabe von ^{14}C-Caprylsäure oder ihres Triglyceride ^{14}C-Trioctanoat die stark erhöhte spezifische Radioaktivität in der Ausatmungsluft vom Maximalwert nur langsam absinkt und den Normalbereich bis zum Ende der Untersuchung übersteigt.

Die ^{14}C-Fettsäure und das ihr entsprechende ^{14}C-Triglyceride verhalten sich in dieser Hinsicht gleichartig, so daß eine erhebliche Beeinträchtigung der Fettverdauung und -resorption oder beider Vorgänge auf Grund dieser Befunde nicht vorzuliegen scheint. In diesem Zusammenhang ist jedoch darauf hinzuweisen, daß GRENIER u. Mitarb. (1968) nach oraler Verabfolgung von ^{14}C-Triolein eine Korrelation der ^{14}C-Radioaktivität im Plasma und im Stuhl mit der spezifischen Radioaktivität in der Ausatmungsluft nicht feststellen konnten. Gleichartige Untersuchungen mit ^{14}C-Caprylsäure oder ^{14}C-Trioctanoat sind noch nicht vorgenommen worden. Der normale Wert oder die nur geringe Steigerung der Fettausscheidung im Stuhl sprechen aber gegen eine Störung der Fettverdauung oder Fettresorption.

Da die Kinetik der spezifischen Radioaktivität erst mehrere Stunden nach Beginn der Untersuchung bei sämtlichen Kranken den Normalbereich übersteigt, ist bei den Kranken eine Beeinträchtigung des Intermediärstoffwechsels zu diskutieren. Veränderungen des Triglyceridpools dürften sich nämlich einige Stunden nach oraler Gabe der ^{14}C-Fettsäure oder des ^{14}C-Triglyceride bei der $^{14}CO_2$-Ausatmung bemerkbar machen. Die Umsatzzeit von Triglyceriden kann mehrere Stunden betragen (FRIEDBERG u. Mitarb., 1961). Andere Verbindungen wie Hydrogencarbonat oder Acetyl-CoA, die im Organismus nach oraler Gabe von Fettsäuren oder Triglyceriden entstehen, unterliegen einem rascheren Stoffwechsel, so daß ihre Auswirkung auf die $^{14}CO_2$-Exhalation während der zweiten Hälfte der Untersuchungszeit hinter dem Einfluß von Triglyceriden zurücktreten dürfte. Die Aussagekraft von $^{14}CO_2$-Exhalationsmessungen wird dadurch eingeschränkt, daß die Ergebnisse auch durch Änderungen von Poolgrößen und von Austauschgeschwindigkeiten zwischen einzelnen Pools beeinflußt werden können. Zur Bestätigung des Verdachts auf eine Beeinträchtigung des Intermediärstoffwechsels sind $^{14}CO_2$-Exhalationsmessungen nach intravenöser Verabreichung der gleichen ^{14}C-markierten Fettsäuren und Triglyceride erforderlich.

Wie bei der Besprechung früherer Resultate unter gleicher Versuchsanordnung betont wurde [GLAUBITT u. FREUDENBERG, 1967; GLAUBITT u. FRAHM, 1968 (1) u. (2)], gestattet die angewandte Methodik nicht quantitative Aussagen über den Anteil des Kohlenhydrat-, Fett- und Eiweißstoffwechsels an der $^{14}CO_2$-Ausatmung oder über die Bevorzugung bestimmter Stoffwechselwege im Intermediärstoffwechsel.

Unsere Befunde demonstrieren, daß unter einer klinisch optimalen Substitutionsbehandlung bei Kranken mit primärem Myxödem bestimmte Stoffwechselgrößen von den Normalwerten abweichen können. Spätere Untersuchungen müssen klären, inwieweit die pathologischen Ergebnisse auf die Grundkrankheit zurückzuführen oder Folge der Substitutionstherapie sind.

Zusammenfassung

Sechs Kranke mit primärem Myxödem unter einer Substitutionstherapie mit Thyreoidea sicca zeigen nach oraler Verabreichung von ^{14}C-Caprylsäure oder ^{14}C-Trioctanoat eine lang anhaltende Erhöhung der spezifischen Radioaktivität in der Ausatmungsluft nach Erreichen des Maximalwertes. Eine Beeinträchtigung des Intermediärstoffwechsels ist zu diskutieren.

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W. BOLT, F. RITZL, H. G. SIEBERTH und H. M. BOLT (Med. Univ.-Klinik, Köln und Institut für Med. der Kernforschungsanlage Jülich des Landes Nordrhein-Westfalen): **Die Ausscheidung von Androgenen und Oestrogenen bei Gesunden und bei Patienten mit Niereninsuffizienz. Untersuchungen mit tritiummarkierten Steroiden**

Um einen Einblick in die Funktion der Nieren im Androgen-Oestrogenstoffwechsel zu erhalten, sollen im folgenden die quantitativen Verhältnisse nach ^{3}H-Testosteroninjektion bei Normalen und bei Patienten mit Niereninsuffizienz miteinander verglichen werden. In diesem Rahmen ist diese Genese der Gynaecomastie bei einem Teil unserer chronisch niereninsuffizienten Patienten von Interesse, die in unserem chronischen Dialyseprogramm in Behandlung stehen [15].

Zunächst sei der Umbau und die Ausscheidung der Sexualhormone bei *Gesunden* betrachtet. Unseren bisherigen Untersuchungen zufolge ergibt sich folgendes Bild [1 bis 3].:

Nach Injektion von tritiummarkiertem Testosteron wird innerhalb von 3 Tagen über 90% der Aktivität im Urin ausgeschieden, davon 80% innerhalb der ersten 24 Std. Die biologische Halbwertszeit des an das Testosteron geknüpften Tritiums beträgt ca. 10 Std. Die Verhältnisse liegen somit anders als bei den gebräuchlichen Laboratoriumstieren, die nach ^{3}H- oder ^{14}C-Testosteroninjektion große Teile der Radioaktivität in den Faeces ausscheiden [7 bis 9].

In früheren Arbeiten [9 bis 12] wurde beim Menschen über eine sehr geringe Radioaktivitätsausscheidung berichtet. Nach HORTON, ROSNER u. FORSHAM [13] liegt die Ursache hierfür in der niedrigen spezifischen Aktivität der von diesen Autoren benutzten Testosteronpräparate. HORTON u. Mitarb. [13] fanden ebenso wie wir eine Radioaktivitätsausscheidung im Harn von über 90% der injizierten Aktivität. In den Faeces erscheinen nach Injektion von markiertem Testosteron weniger als 5% der Radioaktivität [9].

Die im Urin nachweisbare Aktivität besteht beim Menschen zu etwa 45% aus 17-Ketosteroiden, zu 5% aus Androstandiolen und bis zu 1% aus Oestrogenen.

ИССЛЕДОВАНИЕ ОРГАНИЧЕСКИХ КИСЛОТ ЭФИРНОГО МАСЛА ЧАЯ

51

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Первое исследование химического состава эфирного масла чая было проведено голландским ученым Ван Ромбургом (цит. по [1]). Им же установлено, что в состав эфирного масла, полученного после ферментации, кроме других веществ, входит салициловая кислота в виде сложного эфира метилсалицилата.

Более подробное исследование эфирного масла зеленого чайного листа проведено Такей и Сакато [2], а черного чая — Ямамото и Като [3, 4].

Согласно данным этих авторов, в эфирном масле зеленого чайного листа было обнаружено присутствие уксусной, пропионовой, н-масляной, изомасляной, изовалерьяновой и капроновой кислот, а в кислотной части эфирного масла черного чая были выделены пропионовая, изовалерьяновая, н-капроновая и пальметиновая кислоты.

В 1939 г. в Химическом институте Академии наук Грузинской ССР Гегеле, Кикодзе и Рухадзе [5] получили эфирное масло из ферментированного грузинского чая. Они определили основные физико-химические константы этого масла, но не провели исследование отдельных его компонентов.

В 1956 г. Яманиши, Такасаки, Курито и Цуджимура [6], исследуя кислотную часть эфирного масла зеленого чайного листа, констатировали присутствие следующих предельных кислот: уксусной, пропионовой, н-масляной, изомасляной, изовалерьяновой и капроновой, т. е. подтвердили данные Такей и его сотрудников.

Как видно, приведенные исследования касаются эфирного масла зеленого чайного листа. Настоящая работа посвящена исследованию кислот как свободных, так и входящих в эфиры эфирного масла черного байхового чая при его производстве и хранении.

ЭКСПЕРИМЕНТАЛЬНАЯ ЧАСТЬ

В наших исследованиях качественное определение органических кислот проводили методом бумажной хроматографии. Эфирные масла были получены по ранее принятой нами методике [7] из

50-56

Biokhim. Prog. Tekhnol. Chain. Proizvod. Akad. Nauk SSSR. Inst. Biokhim, 1966

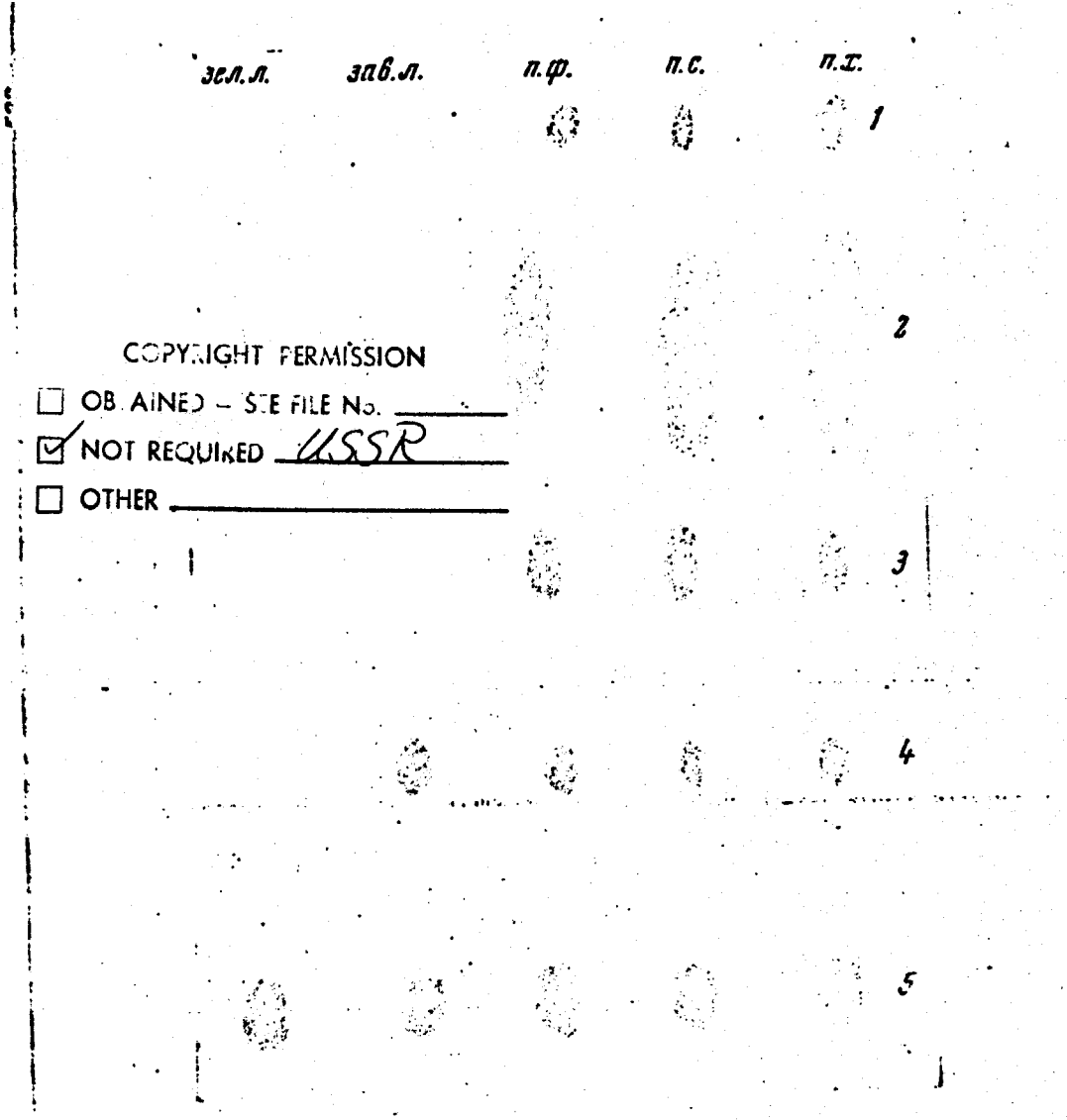

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Рис. 1. Хроматограмма свободных кислот

Зел. л. — зеленый лист; зав. л. — завяленный лист; п. ф. — после ферментации; п. с. — после сушки; п. х. — после хранения; кислоты: *1* — каприловая, *2* — капроновая; *3* — валерьяновая; *4* — мисляная; *5* — уксусная

зеленого и завяленного листа, после ферментации и сушки и после шестимесячного хранения полуфабриката.

Хроматографирование низших жирных кислот (C_1 — C_6) проводили в виде их натриевых солей, а хроматографирование ароматических и высших жирных кислот (выше C_6) — не переводя их в натриевые соли.

С целью идентификации свободных кислот определенное количество эфирного масла чая переносили в делительную воронку и добавляли 5%-ный раствор бикарбоната натрия до создания явно щелочной реакции. После нескольких минут взбалтывания водный слой отделялся. Такую обработку бикарбонатом натрия повторяли три раза. Полученный водный слой дважды обрабатывали диэтиловым эфиром (для удаления следов эфирного масла), затем переносили в фарфоровую чашку и выпаривали до минимального объема. Полученный раствор использовали для хроматографического разделения свободных кислот в виде натриевых солей [8].

На полоски хроматографической бумаги марки Ленинградская-Б при помощи капилляра наносили растворы солей (обычно по 4—5 капель). После этого бумагу помещали на 6 час. в камеру, насыщенную парами смеси н-бутанола +1,5 н. NH_4OH (1 : 1), и хроматографировали в течение 18 час.

После высушивания бумаги на воздухе кислоты были идентифицированы опрыскиванием водным раствором хлорфенолкрасного (150 *мг* хлорфенолкрасного растворяли в 250 *мл* воды и к полученному раствору приливали по каплям 0,5 н. раствор NaOH до малинового окрашивания). Кислоты проявлялись в виде ярко-желтых пятен на фиолетовом фоне. Некоторые из них быстро исчезали.

При этих условиях R_f уксусной и муравьиной кислот очень близки [8], поэтому их разделение проводили, опрыскивая хроматограмму раствором азотнокислого серебра (0,1 н. раствор $AgNO_3$ смешивали с 5 н. NH_4OH в отношении 1 : 1). При высушивании муравьиная кислота немедленно проявляется в виде темно-коричневого пятна, а уксусная не дает такого окрашивания.

Идентификацию свободных кислот проводили по совпадающему R_f соответствующих солей кислот метчиков.

Приведенные данные (рис. 1) показывают, что в эфирном масле зеленого чайного листа обнаруживается присутствие только уксусной кислоты. В завяленном листе появляется и масляная кислота.

К концу ферментации обнаруживаются, кроме указанных кислот, валерьяновая, капроновая и каприловая кислоты. Состав их во время сушки чая не меняется, но, судя по объему площади пятен, замечается некоторое увеличение капроновой кислоты.

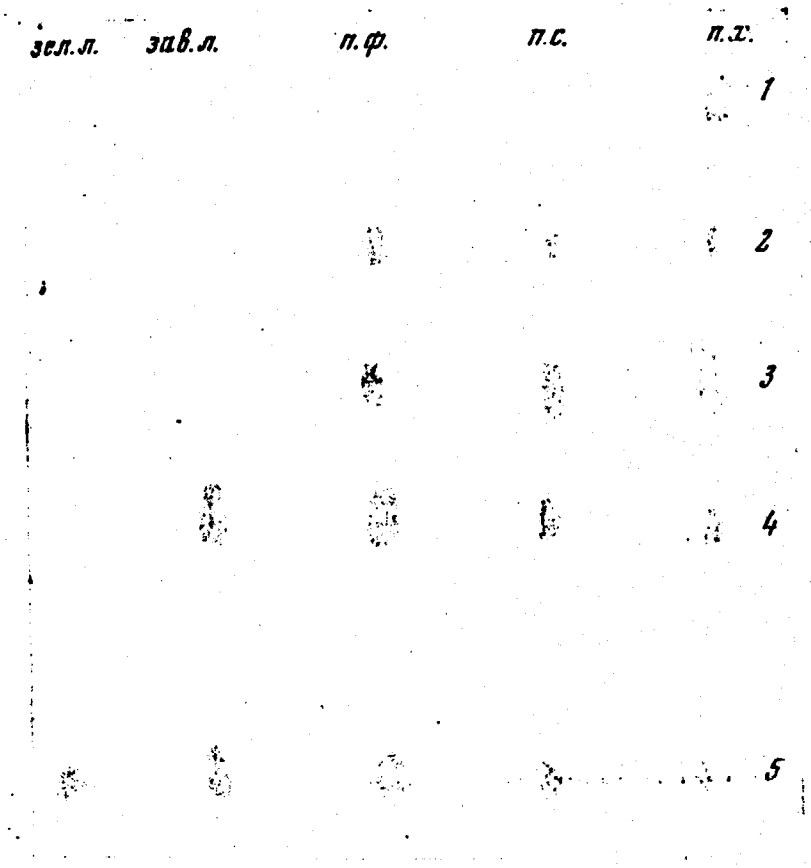

Рис. 2. Хроматограмма кислот, входящих в эфир

Кислоты: *1* — каприловая; *2* — капроновая; *3* — салициловая; *4* — бензойная; *5* — уксусная. Остальные обозначения те же, что на рис. 1

Для большого уточнения полученных результатов мы попытались идентифицировать указанные кислоты, не переводя их в соли. Для этого к раствору солей добавляли 0,5 н. раствор серной кислоты до появления явно кислой реакции. Полученную смесь трижды взбалтывали с диэтиловым эфиром, эфир выпаривали под вакуумом, остаток растворяли в этаноле. Полученный раствор наносили (5—7 капель) капилляром на бумагу марки Ленинградская-Б. Хроматографирование проводили в той же смеси, что и для натриевых солей этих кислот [8].

Для идентификации хроматограмму опрыскивали смесью спиртовых растворов метилового красного и бромфенолового с двойным объемом фосфатного буфера (M/15 Na_2HPO_4).

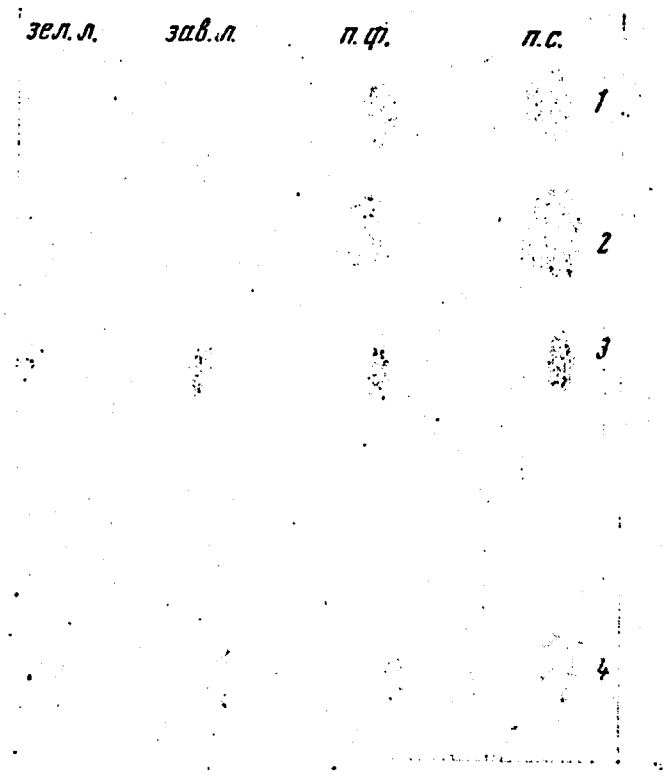

Рис. 3. Хроматограммы гидроксамовых кислот

Обозначения те же, что на рис. 1

С целью исследования кислот, входящих в эфиры, образцы эфирного масла после обработки раствором бикарбоната натрия, т. е. после удаления свободных кислот, переносили в круглодонную колбу, добавляя 0,5 н. раствор едкого натра, и кипятили в течение 2 час. Смесь после омыления переносили в делительную воронку и экстрагировали по три раза диэтиловым эфиром. Водный слой выпаривали в фарфоровой чашке до небольшого объема. Хроматографическую идентификацию кислот, входящих в эфиры, проводили как в виде солей, так и в виде кислот по описанной выше методике.

На рис. 2 приводится результат идентификации кислот эфиров с соответствующими метчиками.

Из этих данных видно, что в эфирном масле зеленого чайного листа находится только эфир уксусной кислоты. В процессе завяливания образуется эфир бензойной кислоты.

Во время ферментации чая, когда особенно энергично идет процесс этерификации, дополнительно образуются эфиры салициловой и капроновой кислот. При сушке сохраняются эфиры всех кислот, но их количество уменьшается, что, в конечном счете, дает уменьшение эфирного числа. Во время хранения чая не замечается исчезновения эфиров каких-нибудь кислот, наоборот, за тот срок, при котором хранились наши опытные чаи, замечалось образование в некоторых образцах эфира каприловой кислоты.

Однако уменьшение эфирного числа вызвано, по всей вероятности, количественным уменьшением эфиров отдельных кислот.

Для большей уверенности в правильности полученных результатов эфиры, входящие в эфирное масло чая, были идентифицированы путем превращения в соответствующие гидроксамовые кислоты с дальнейшим их хроматографированием.

Хроматографическое разделение эфиров в виде гидроксамовых кислот проводили по следующей методике [9]. Несколько капель эфирного масла переносили в пробирку, туда же прибавляли 10 *мл* диэтилового эфира и 2,5 *мл* щелочного раствора солянокислого гидроксиламина (смешивали равные объемы 5%-ного раствора солянокислого гидроксиламина и 12,5%-ного раствора едкого натра в метаноле; выделившийся при этом хлористый натрий отфильтровывали). Смесь выдерживали 30 мин. при 25°, после чего добавляли 0,2 *мл* концентрированной HCl. Образовавшийся при этом осадок отфильтровывали. Эфирный экстракт сгущался в вакууме до 2—3 *мл*. Сгущенный раствор при помощи капилляра наносили (5—7 капель) на хроматографическую бумагу марки Ленинградская-Б. В качестве растворителя применяли н-амиловый спирт, уксусную кислоту и воду (4 : 1 : 5).

Обнаружение пятен гидроксамовых кислот проводили опрыскиванием 5%-ным раствором треххлорного железа в метаноле, подкисленного концентрированной соляной кислотой.

После высушивания при температуре 60° появлялись фиолетовые пятна на желтом фоне. Поскольку мы не располагали эфирами всех предполагаемых кислот, полученные пятна не сравнивались с метчиками, но в результате многократного повторения было установлено, что число пятен эфиров по гидроксамовым кислотам (рис. 3) совпадает с числом пятен кислот, полученных после омыления (см. рис. 2).

Этим подтверждаются наши выводы о кислотном составе эфиров эфирного масла чая.

Итак, подводя итоги изложенному, можно сделать вывод, что состав свободных кислот и кислот сложных эфиров, входящих в эфирное масло, претерпевает существенные изменения в процессе переработки черного байхового чая.

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ИССЛЕДОВАНИЕ ТЕРПЕНОВЫХ И АРОМАТИЧЕСКИХ СПИРТОВ В ЭФИРНЫХ МАСЛАХ ЧАЯ МЕТОДОМ ТОНКОСЛОЙНОЙ ХРОМАТОГРАФИИ

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Из класса веществ, входящих в эфирное масло пищевых продуктов, спиртам принадлежит существенная роль. Установлено, что в создании приятного аромата участвуют в основном спирты терпенового и ароматического ряда. Поэтому, исследование этих спиртов в эфирных маслах черного байхового чая имеет большое теоретическое и практическое значение.

Ван Ромбург (цит. по [1]) первый установил в эфирном масле чая присутствие метанола в виде сложного эфира салициловой кислоты.

В дальнейшем Гильдемейстер и Гоффман [2] обнаружили в эфирных маслах чая наличие β- и γ-гексанола в форме сложного эфира фенилуксусной кислоты. Такей и Сакато [3], исследуя эфирное масло зеленого чайного листа, констатировали присутствие β- и γ-гексанола, *n*-гексанола, бензилового и фенилэтилового спирта, линалоола и гераниола.

Ямамото и Като [4] установили в составе эфирного масла черного байхового чая присутствие в виде эфиров гексанола, нормального октанола, фенилэтилового спирта, гераниола и цитронелоола.

Накабаяси и Макато [5], исследовавшие зависимость аромата черного чая от общего содержания летучих спиртов, не выявили определенной корреляции между ароматом чая и содержанием в нем летучих спиртов. Как видно, указанные исследования спиртов касаются в основном эфирного масла, полученного из зеленого листа и готовой продукции черного байхового чая.

До настоящего времени вопрос образования и превращения эфирного масла по стадиям переработки чая изучался мало. Изучению состава спиртов эфирного масла нашего отечественного чая также уделялось немного внимания. Настоящая работа посвящена выяснению указанного вопроса.

Для исследования эфирное масло было получено по ранее принятой нами методике [6].

Для хроматографирования пользовались нейтральной частью эфирного масла, полученной после омыления [6].

Эфирную часть после омыления переносили в колбу для вакуумной отгонки и эфир отгоняли под вакуумом, оставшийся

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THE RELATIVE RATE OF PENETRATION OF THE LOWER SATURATED MONOCARBOXYLIC ACIDS INTO MAMMALIAN ERYTHROCYTES

53

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FOUR FIGURES

Since the work of Overton (1895, 1899, 1902) the idea has been widely accepted that compounds soluble in lipid solvents penetrate cells by reason of their solubility in the lipids of the cell surface. A number of investigators (Loeb, Harvey, Crozier, Jacobs, Bodansky, Collander and Bärlund, Höber and Orskov, Dziemian) have tested this theory and, in general, the relative rate of penetration of the lipid soluble compound has been found to parallel its distribution in an oil-water mixture. These investigators used various methods to measure penetration of lipid soluble compounds, not all of which are equally reliable. Thus Loeb ('09, '13) using Arbacia eggs, studied the rate of fertilization membrane formation on exposure to the lower fatty acids. He found that as the fatty acid chain was lengthened, the membranes formed more rapidly, and considered his observations to support the Overton theory. However, in the absence of information showing that penetration must occur before membrane formation can take place, the Loeb results are best considered inconclusive support for the theory. Harvey ('14) and Crozier ('16) studied the penetration of acids into tissues of two invertebrate forms

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Cap. Acid

which contained natural indicators. A color change in the indicator was taken as evidence of penetration of the acid. Both investigators found valeric or isovaleric acid to penetrate more rapidly than lower acids.

The studies of Jacobs ('26), Bodansky ('28), Höber and Orskov ('33) and Dziemian ('39) were made on erythrocytes using hemolysis as a criterion of penetration. This method has many advantages when used critically but, as will be shown by this report, cannot be accepted as a measure of penetration of compounds which enter cells more rapidly than water, without reservation. Of this group of hemolysis studies only those of Jacobs and Bodansky were concerned chiefly with the penetration of fatty acids into erythrocytes. Jacobs, using the ammonium salts of the fatty acids from formic to valeric, was able to show an increasing rate of penetration for this series. This is in agreement with the Overton theory. Bodansky studied the hemolytic effects of the fatty acids themselves. However, he neglected to distinguish between hemolysis wrought by injury and that produced by penetration of the acid and water and it is difficult to evaluate his results. The work of Collander and Bärlund ('33) dealt with the penetration of lipid soluble compounds in the cells of the large alga, Chara. They determined the amount of a compound which had entered the cell by chemical analysis and found for the most part agreement between the rate of penetration and the lipid-solubility of the compound. They did not study fatty acid penetration.

The present study has attempted to measure the relative rate of penetration of the lower fatty acids into mamalian erythrocytes by a new method. This method is essentially chemical and depends upon the fact that oxyhemoglobin when placed in an environment containing an increased hydrogen ion concentration loses some of its oxygen. This loss of oxygen may be determined spectrophotometrically. Under experimental conditions the rate at which oxyhemoglobin is converted into reduced hemoglobin in the intact erythrocyte in a fatty acid environment may be used as a measure of the rate

of fatty acid penetration into the erythrocyte. By the use of this method the rate at which the lower fatty acids, from formic through caprylic, penetrated beef and human erythrocyte was measured. It will be shown that the penetration rate was slowest for formic acid, increased to valeric acid and then again decreased in such a manner that heptylic and caprylic acids were found to penetrate more slowly than butyric acid. Lytic hemolysis studies were done with the Parpart densimeter ('35) and it was found that the results obtained in this way differed from those penetration data obtained by the spectrophotometric method.

METHODS

Since the work of Hüfner (1879) the absorption spectrum of oxyhemoglobin has been known and with instrumental improvement has been measured with increased accuracy down to the present time (Hari, '17; Drabkin, '35; Rubinstein and Ravikovich, '46). In a dilute suspension of erythrocytes oxyhemoglobin may be shown to change its absorption characteristics, as measured spectrophotometrically, by lowering the oxygen tension of the environment or by increasing the hydrogen ion concentration in the interior of the cells. This latter finding by Barcroft and Murray ('22) provides a means of measuring by the use of a spectrophotometer the relative amount of acid which has penetrated a suspension of erythrocytes. Hartridge and Roughton in a series of studies ('23, '25) have shown that under their conditions of measurement the dissociation of oxyhemoglobin at lower pH values is nearly complete within 1/50 of a second. This information makes feasible the measurement of the rate of penetration of acid compounds into erythrocytes provided such penetration occurs more slowly than the limiting dissociation time of oxyhemoglobin.

A device was made for the rapid mixing of red cell suspension and acid solutions. A drawing of this appears in figure 1. It consists essentially of two reservoirs, 6 and 7

which empty through stopcocks, 8 and 9 either through the exit tubes 10 and 11 or through the master stopcock, 12, into a mixing chamber, 13. The mixing chamber, shown in the inset, is so constructed that complete mixing occurs within it and a homogeneous mixture runs into the observation tube, 14. The entire device was made of pyrex glass. For experi-

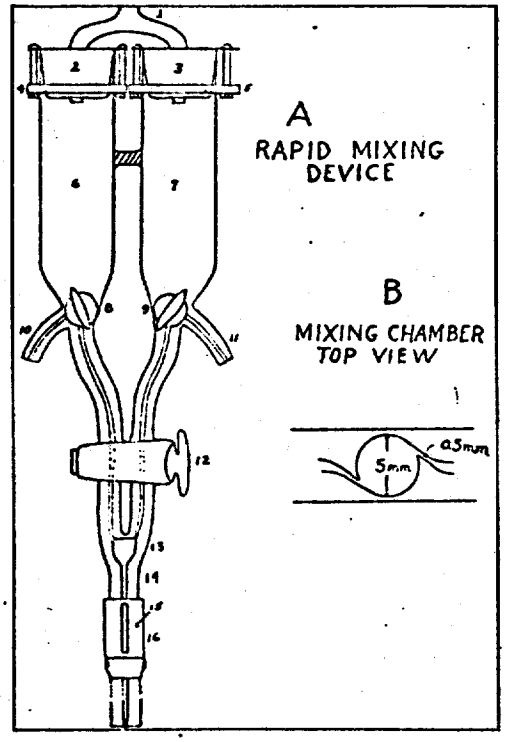

Fig. 1 Sketch of rapid-mixing device.

mental use the rapid mixing device was mounted above a Coleman, model 10S, spectrophotometer in such a manner that the observation tube passed vertically through the cuvette holder of the spectrophotometer. The monochromatic beam of light from the spectrophotometer then passed through the lumen of the observation tube. In order to focus the monochromatic beam to insure its passage through the lumen of

the tube, a glass collar (16 in fig. 1) was made of optically flat glass sealed together with DeKhotinsky cement and held in place on the observation tube by a piece of rubber tubing which readily permitted changing its position as experiment required. A piece of thin brass shim containing a longitudinal slit the same width as the lumen of the observation tube and the same length as the beam of light from the spectrophotometer was inserted inside the glass collar on the exit side of the light beam. When in use the glass collar was filled with glycerol which has approximately the same index of refraction as does pyrex glass.

When a red cell suspension is placed in one reservoir of the rapid mixing device and a dilute solution of a lower fatty acid in the other, the two liquids will come together in the mixing chamber when the large stopcock is opened. From here they pass through the observation tube, traveling enroute through the monochromatic beam of the spectrophotometer. It is apparent that the time which has elapsed from initial contact in the mixing chamber until the mixture passes through the center of the monochromatic beam represents the reaction time or the time in which the acid has had opportunity to enter the cell and bring about a dissociation of the oxyhemoglobin to a new equilibrium. Experimentally a measurement is made of the amount of light transmitted through the observation tube when the mixture is moving, i.e., a flow reading, and when it is stopped, i.e., a static reading. The difference between the two readings is taken as a relative measure of the acid which penetrated the cells in a given reaction time. The reaction time was varied by changing the mixing distance, i.e., the distance from the mixing chamber to the center of the monochromatic beam of the spectrophotometer, or by changing the speed of flow of the liquids leaving the reservoirs. The latter object was accomplished by fastening two stoppers (2 and 3 in fig. 1) to the tops of the reservoirs and connecting the reservoirs to a compressed air source. By calibration the reaction times for a series of mixing distances

and pressures were determined. The following equations were used in the calculation of a given reaction time:

$$\text{L.F. (cm/sec)} = \frac{\text{V(cm}^3\text{/sec)}}{\text{A(cm}^2\text{)}} \qquad (1)$$

This equation states that the linear flow (L.F.) is equal to the volume (V) of liquid in milliliters per second passing through the observation tube divided by the area (A) of the lumen of the observation tube.

$$\text{R.T. (sec)} = \frac{\text{D(cm)}}{\text{L.F. (cm/sec)}} \qquad (2)$$

This second equation states that the reaction time (R.T.) measured in seconds is equal to the mixing distance (D) in centimeters divided by the L.F. obtained from the first equation.

Beef blood was obtained from one of the local slaughter houses and human blood was secured from laboratory personnel. Both types of blood were citrated at the time of collection to prevent clotting and were subsequently strained through sterile gauze to remove any clots which may have formed. Whole blood was used in the preparation of the red cell suspensions in concentrations of 1 to 1.5% blood in 1% NaCl solutions. Because of the initial acidity of the saline solutions a small amount of NaOH was added to bring the pH of the red cell suspension to approximately 7.4. Buffer solutions were not used because the method depended upon changing the pH of a red cell suspension from about 7.4 to 4.2. Further, one wished to study the penetration of the acid molecule and not the acid anion. The lower members of the saturated, monocarboxylic acid series from formic through caprylic acid were studied. Stock molar solutions of formic, acetic, propionic and butyric acids were prepared and appropriate dilutions made from these. Because of the relative insolubility of valeric, caproic, heptylic and caprylic acids, dilutions were prepared directly from the concentrated acid. Dilute acid solutions were prepared and used on the same day. Table 1 lists the acid concentrations used with the ap-

TABLE 1

ACID	MOLAR CONC. OF ACID IN DILUENT	ESTIMATED CONC. OF ACID IN FINAL MIXTURE	AVER. pH VALUE OF MIXTURE	RATIO OF SALT/ACID AT AVER. pH	SURFACE [1] TENSION DYNES/CM	MR_D [2]	DISTRIBUTION [3] RATIO
Formic	0.006	0.003	4.34	4.68/1	72.51	8.55	0.005 (0.1 N acid)
Acetic	0.008	0.004	4.18	0.282/1	70.78	12.98	0.03 (0.1 N acid)
Propionic	0.01	0.005	4.26	0.255/1	66.21	17.52	0.1515 (0.01 N acid)
Butyric	0.01	0.005	4.28	0.282/1	56.58	22.05	0.443 (0.01 N acid)
Valeric	0.009	0.0045	4.26	0.291/1	65.56	26.3	2.60 (0.01 N acid)
Caproic	0.01	0.005	4.27	0.257/1	55.58	30.75	9.47 (0.01 N acid)
Heptylic	0.008	0.004	4.39	0.358/1	40.25	35.7	49.3 (0.01 N acid)
Caprylic	0.003	0.0015	4.88	1.09/1	28.82	39.7	31.9 (0.001 N acid)

[1] Measured against air.
[2] Molecular refraction for Na line D.
[3] Ratio for acid in olive oil to acid in water at equilibrium (Bodansky, '28).

proximate concentration obtaining in the red cell suspension upon dilution in the rapid mixing device. The acid concentration used was determined by experiment as that concentration which upon mixing with the red cell suspension would yield a pH of approximately 4.2. At this pH the erythrocytes were not immediately hemolyzed and the hydrogen ion concentration of the environment had been appreciably increased. Caprylic acid solutions were used at a slightly higher pH because of their lytic action and slight solubility.

It was found experimentally and could be expected from an examination of the oxygen dissociation curve that for a given hydrogen ion concentration a greater amount of oxyhemoglobin dissociated at oxygen tensions below 60 mm of Hg than at oxygen saturation. In practice beef red cell suspensions and acid solutions were equilibrated to calculated oxygen tensions of 10 mm Hg and human red cells suspensions to 20 mm Hg. The equilibration was accomplished by mounting two rotating tonometers on the laboratory table beside the spectrophotometer. Water displaced the gas mixture by means of a siphon and drove the gas through the tonometers from which it was carried through the reservoirs of the rapid mixing device and finally through a water trap. After equilibration, which was usually accomplished within 25 minutes, the red cell suspension and the acid solution, each of approximately 100 ml, were passed directly into the reservoirs without contact with air. The tonometers were not placed in a constant temperature bath but the room in which the equilibration was done maintained a fairly constant temperature. During any one experiment the temperature was never observed to vary more than 0.5° C. and throughout the period of this study the room temperature ranged from 20° to 23° C.

Following equilibration and transfer of the red cell suspension and acid solution to the reservoirs of the rapid mixing device, a "run" was made. This consisted of 5 or 6 different observations of flow and static transmission values at different reaction times. The mixing distance was maintained constant during a run and the reaction time was varied

by changing the speed of flow of the liquids through the observation tube. In a series of three runs at the same reaction time the difference between flow and static readings, measured as densities (in which the optical density is the reciprocal of the logarithm of the per cent light transmitted), were averaged. These average values were then corrected to a scale of 100 in order to make the different series comparable. Control studies were made using 1% NaCl solutions in place of the acid diluent, in order to determine instrument variation, effects of change of velocity of flow and turbulence. The effects of light scatter were also investigated and considered of negligible importance in this study. When a red cell suspension was made up in 1% NaCl solution and a series of readings on the suspension made, it was found that consecutive readings did not vary more than 0.5%. Concentrations of red cells were used which transmitted approximately 50% of the incident light. Under these conditions, approximately 5% difference in transmission of light occurred between the absence of penetration of an acid and maximum penetration.

It was of importance to this study to know when hemolysis began and something of its course in time. Also because hemolytic techniques had been used as a measure of the penetration of compounds into red cells it was of interest to compare hemolytic data for these acids with the penetration data obtained by the spectrophotometric technique. Therefore, studies with the densimeter apparatus of Parpart ('35) were made for each acid and each type of blood studied using the same acid and blood concentrations as were used in the rapid mixing device.

RESULTS

A summary of typical results obtained is shown in figure 2 for beef blood and figure 3 for human blood. These graphs are semi-logarithmic plots in which the lines were fitted by the method of least squares. Each point on a line is an average of three measurements. The ordinate, corrected density difference, is plotted against time in seconds. The time

referred to is the reaction time previously described. Density differences were obtained by subtracting static density values from flow density values. An average of three such differences for a given reaction time was corrected on the basis of 100 in order to bring the results for different acids on one graph. The failure of the lines for the different acids to have a common origin in both figures 2 and 3, is owing to their having been calculated lines. While the exponential nature of these data may be expected from theoretical considerations,

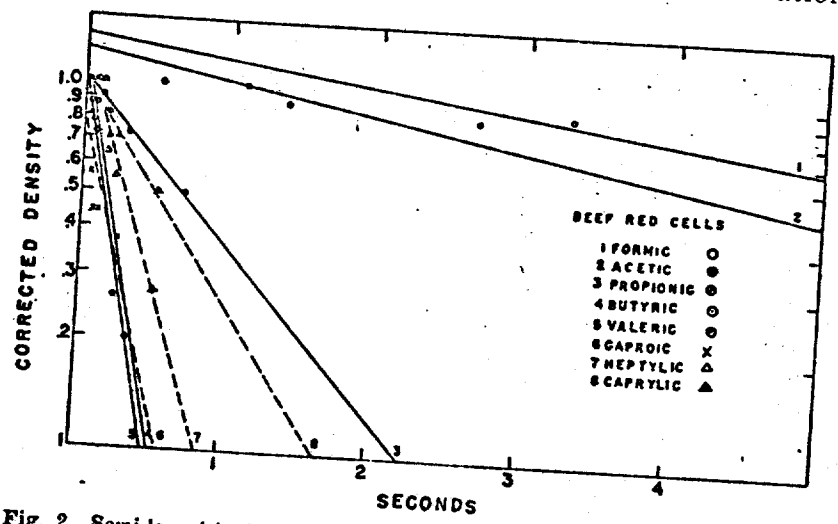

Fig. 2 Semi-logarithmic plot of representative data for beef erythrocytes. The ordinate, corrected density, refers to density differences between flow and static readings in the apparatus.

it was confirmed by plotting the corrected density difference against the reaction time. For convenience of presentation the semi-logarithmic plot only is given here.

Figure 2 shows a progressive increase in the rate of penetration of the acids into beef cells from formic to valeric, with butyric, valeric and caproic acids having much the same slope. The slopes of these three acids are not statistically different. A reverse condition was obtained for the rate of penetration of heptylic acid which was slower than caproic while caprylic

acid is slower than heptylic. Figure 3, for human blood, shows much the same trend as figure 2 for beef blood. The exception to be noted is that caprylic acid has a slightly slower rate of penetration than does heptylic acid. Since the lipid solubility of these acids increases with the length of the carbon chain, the results obtained with heptylic and caprylic acids were sufficiently in disagreement with the Overton theory to suggest a confirmatory study. This was undertaken with a Beckman spectrophotometer which has an altogether different optical and recording system from that of the Coleman 10S.

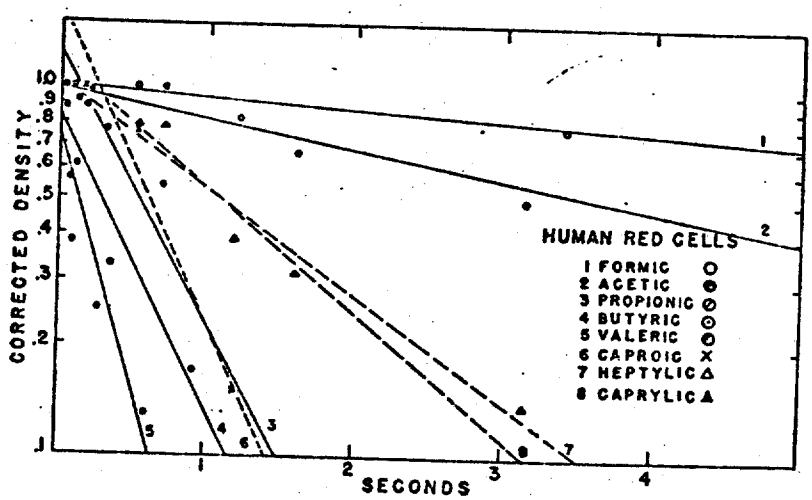

Fig. 3 Semi-logarithmic plot of representative data for human erythrocytes.

The same results were obtained with the Beckman instrument using beef blood only.

The results of the densimeter studies, which were performed for each acid and each type of blood, are plotted in figure 4. These graphs compare time to 50% hemolysis, using the densimeter technique, with time to 50% penetration, using the spectrophotometric technique. The data for the penetration plots were calculated from the relation, $\log C/C_0 = -kt$, where C_0 is the final concentration of acid at equilibrium inside the cells, C, the concentration at any given time, and

$-k$, the slope of the plot of log C/C_0 against t, the time in seconds. When $C = 50$, $C_0 = 100$, and $t = 50$, the following relations exist: $t_{50} = 0.3010/-k$. An examination of figure 4 reveals that the beef and human red cells produce parallel curves for the hemolytic data and parallel curves for the

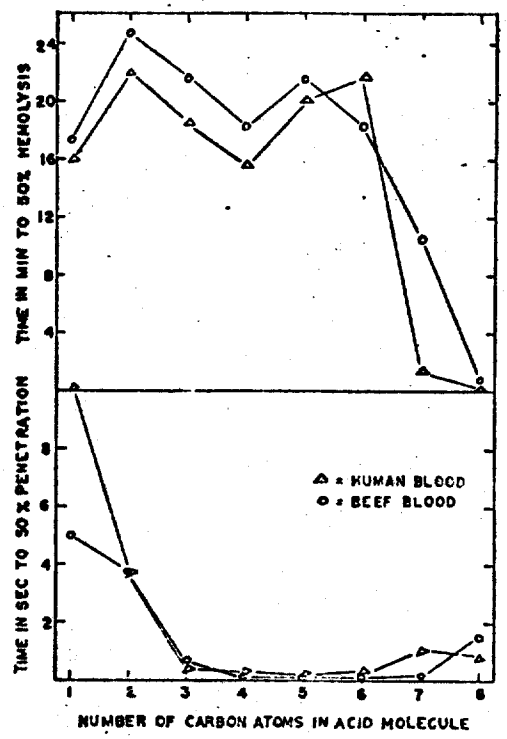

Fig. 4 Comparison between hemolytic and penetration times for each of the acids studied. The hemolysis scale is in minutes and the penetration scale in seconds.

penetration data. However, the two sets of curves are roughly the inverse of each other. In the hemolytic curves, formic acid produces 50% hemolysis about as rapidly as butyric, and one might expect that valeric would be faster than either but this is not the case. In the penetration data the most unexpected result is the decrease in the rate of penetration with

heptylic and caprylic acids when these are known to be the most lipid soluble of the group studied. The times to 50% penetration are listed in table 2 for each acid and each type of blood studied.

TABLE 2

Time to 50% penetration

ACID	BEEF CELLS —k[1]	BEEF TIME IN SEC. TO 50% PENETRATION	HUMAN CELLS —k	HUMAN TIME IN SEC. TO 50% PENETRATION
Formic	0.05969	5.04	0.02964	10.16
Acetic	0.07934	3.79	0.08073	3.73
Propionic	0.4564	0.66	0.7316	0.412
Butyric	2.0816	0.145	0.7949	0.379
Valeric	2.080	0.145	1.3852	0.2175
Caproic	1.5312	0.197	0.8372	0.359
Heptylic	1.2558	0.239	0.2895	1.04
Caprylic	0.6075	0.496	0.3424	0.878

[1] — k = slope of plot from figure 2.

DISCUSSION

A number of investigators have studied the penetration of the lower fatty acids into cells and a few have determined the relative order of penetration of these acids. Harvey ('14a, '14b), taking advantage of a natural indicator in the pigment cells of the holothurian, Stichopus, observed the following order of penetration of the lower fatty acids: valeric = formic > butyric = propionic = acetic. Crozier ('16a, '16b) reported similar results using pigment cells in a mollusc, Chromodoris. He obtained the series: isovaleric > formic > butyric > propionic > acetic. Crozier later ('18) determined the effect of these acids on the sensory activation of the earthworm, Allolobophora, but these results, like those obtained earlier by Loeb ('09, '13) on sea urchin fertilization membrane formation or those of Taylor ('28) on taste threshold of acids in human beings, are difficult to assess because of the absence of evidence for penetration.

Gryns (1896) observed that the ammonium salts of acetic, propionic and butyric acids penetrated horse erythrocytes. Later Jacobs ('26, '27) measured the hemolysis times of ammonium formate, acetate, propionate, butyrate and valerianate in beef erythrocytes. He found an increase in the rate of hemolysis with increasing length of the carbon chain, although some leveling off in the speed of penetration was observed in the butyrate and valerianate salts. He advanced evidence that the hemolysis was produced by the penetration of ammonia and fatty acid molecules following hydrolysis of the salt. Subsequently Jacobs and Parpart ('38) and Jacobs ('40) were able to show that at extreme pH values, either acid or alkaline, the hemolysis was slower than in the neutral range when larger numbers of both species of molecules were available for penetration. Höber ('36) made a study of the hemolysis rate of the sodium and ammonium salts of the lower fatty acids using the photocell method of Höber and Orskov ('33). He found, using red cells of the beef, sheep, rat, man and mouse, that exposure of these cells at pH 7.3 to solutions of the sodium salts of the C_2 to C_6 fatty acids resulted in no detectable alteration of the cells within many hours; however, the C_7 to C_9 fatty acids produced hemolysis within 20 minutes. With the ammonium salts of these acids he obtained the same results as did Jacobs. He further observed the hemolytic rate for the ammonium salts rose among the species studied in the following order: mouse, rat, man < beef < sheep. Dziemian ('39) who repeated this work in part, using the ammonium salts of the C_1 to C_4 fatty acids, was in agreement with it except for some of the species difference observations. He found the rat cells more permeable than the beef cells in every instance studied.

Stewart ('31) investigated the volume changes in sea urchin eggs when exposed to the same series of ammonium salts of fatty acids as Jacobs had studied in beef erythrocytes. She also found an increasing rate of volume change with increasing carbon content of the fatty acid and attributed the differential rate of penetration to the relative ease of solubility

of these acids in the lipids of the egg surface. Bouillenne in 1930 made a study of the penetration of the ammonium salts of the lower fatty acids into epidermal cells of Tradescantia virginica using a deplasmolysis technique. He observed the same order of penetration as had Jacobs and Stewart. In these studies of Jacobs, of Höber, of Stewart and of Bouillenne, the penetration of ammonia molecules must be regarded as a contributing factor in the volume change or hemolysis observed. And while there is no reason to believe that in the absence of ammonia the order of penetration of these acids would be changed, the rate of hemolysis or volume change may be modified. Bodansky ('28, '31, '32, '33) investigated this point in dog and human erythrocytes using solutions of the lower fatty acids in physiological salt solutions. With complete hemolysis as a criterion of penetration rate, he observed an increase in rate of penetration from C_2 to C_{10} fatty acids. He correlated these findings with the relative solubility of these acids in olive oil and associated their penetration into the cells with lipid solubility. Solutions of the acids C_2 to C_6 were used at a pH below 4.0; the solutions of C_7 to C_{10} acids are intensely lytic as shown by Höber ('36). This work of Bodansky's appears to involve what Jacobs ('24) called secondary penetration of acids or penetration with injury. His work does raise the question as to what extent hemolysis may be used as a criterion of penetration of a lytic compound. The work of Parpart and Shull ('35) indicated that in studies with non-lytic compounds such as glycerol, volume changes are closely paralleled by entrance of the glycerol. In the present study a comparison was made between rates of penetration as determined by hemolysis, using the densimeter technique of Parpart ('35) and penetration, as determined spectrophotometrically by the modified Hartridge-Roughton technique. The results are shown in figure 4. The irregularity in the hemolysis curve for the C_4 acid may be owing to evaporation of fluid in the densimeter. However, the hemolysis curves bear no resemblance to the penetration curves, although aside from instrumental differences both curves were

obtained under the same conditions. This graph indicates that rates of hemolysis of red cells by lytic compounds do not parallel rates of penetration of these comopunds as determined by the modified Hartridge-Roughton technique.

The lower graphs of figure 4 show clearly the results of this investigation on beef and human cells. An increase in rate of penetration occurs between C_1 and C_5 acids with C_4, C_5 and C_6 acids exhibiting a nearly constant rate of penetration. The C_7 and C_8 acids penetrate more slowly than the C_4 to C_6 acids. The fact that these studies were made at the pH range 4.2 to 4.8 rather than at neutrality may have influenced the results. However, it was technically impossible to conduct the experiments af pH 7.0 and in any case, the measurements were made in such short periods of time that the lytic action of the higher acids was minimized. In addition to presenting information on the relative rate of penetration of these acids the time to 50% penetration has been calculated and has been found to be quite rapid, varying from 0.14 seconds to over 5.0 seconds as shown by table 2. The technique used in the present study is thought to yield less equivocal data on the penetration of these acids than any of the other methods used in this type of investigation. The fact that the present study has been limited to the blood of two species of mammals and has been made at approximately a single pH value limits the general applicability of the results. However, the results obtained, when their limitations are considered lend only partial support to the published evidence for a continuous series of fatty acids whose rate of penetration into red cells increases with increasing carbon content of the fatty acid.

Some of the physical characteristics of the acids studied in this investigation are listed in table 1. An attempt has been made to correlate the penetration results of this study with some of these characteristics, particularly with concentration, molecular volume or distribution ratio. The C_1 to C_5 acids do appear to penetrate in the order of their relative solubility in olive oil but not in proportion to their solubility. The rates of penetration of the C_6 to C_8 acids do not follow

their relative solubility in olive oil. These data on penetration do not consistently favor the idea that these acids penetrate by reason of their solubility in lipid regions of the erythrocyte and in this respect support the findings of Dziemian ('39). They do suggest that molecular size, in the case of the C_6, C_7 and C_8 acids, may be a limiting factor in the rate of penetration. And it is suggested that the plateau observed in butyric, valeric and caproic acids may represent a balance of two opposite forces, increasing molecular volume, on the one hand, tending to retard penetration and increased solubility in lipid regions, on the other hand, tending to increase the penetration rate.

In considering the significance of the results of this investigation, the fact should be noted that the times for penetration of these acids were measured and were found to be, by comparison with hemolytic times, of a much smaller magnitude. Future work with the method described in this paper should provide information leading to the determination of permeability constants for these acids.

SUMMARY

1. A modification of the Hartridge-Roughton rapid mixing apparatus was developed and used in conjunction with a spectrophotometer to measure the relative rates of penetration of lower fatty acids into beef and human erythocytes. The measurements of penetration depended upon the experimental observation that oxyhemoglobin dissociates to a new equilibrium with an increase in the hydrogen ion concentration within the cell.

2. The relative rates of penetration of the fatty acids investigated were found to be (a) for beef cells: caprylic < heptylic < caproic = valeric = butyric > propionic > acetic > formic; (b) for human cells: caprylic = heptylic < caproic < valeric > butyric > propionic > acetic > formic.

3. The rates of hemolysis by these acids were determined and found to be (a) for beef cells: caprylic > heptylic >

caproic > valeric < butyric > propionic > acetic < formic; (b) for human cells: caprylic > heptylic > caproic < valeric < butyric > propionic > acetic < formic.

4. The times to 50% penetration of these acids were measured and found to be of much smaller magnitude than those determined by hemolytic methods.

5. The results of penetration and hemolysis are discussed in the light of previous hemolytic work done with these acids and the more general studies made of penetration into eggs and tissue cells.

6. An attempt was made to correlate the penetration results with lipid solubilty, surface tension or molecular volume data. The retardation of penetration observed in the C_6, C_7, and C_8 acids suggest that in this homologous series in which lipid solubility increases with increasing carbon content of the acids, molecular volume remains a limiting factor in the penetration rate.

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Metabolism of 1-C^{14} Octanoic and 1-C^{14} Palmitic Acid by Rat Intestinal Slices.* (30565)

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The classic studies of Bloom and Chaikoff (1,2) and Borgström(3) demonstrated that after intestinal absorption, fatty acid molecules with chain lengths of 10 or less carbon atoms were transported primarily in the portal vein whereas fatty acids with chain lengths of greater than 16 carbon atoms were transported predominantly in lymph. Recent studies(4-7) have indicated that differences in the behavior of fatty acids during absorption are probably due to variations in their intramucosal metabolism as well as to differences in physical properties. However, these studies have dealt primarily with the processes of mucosal hydrolysis of glycerides and mucosal esterification of fatty acids. Little attention has been directed at the problem of catabolism of fatty acids by intestinal mucosa.

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In the present investigation studies were undertaken to define more clearly the differences in the mucosal metabolism of a long chain fatty acid (palmitic) as compared to a medium chain fatty acid (octanoic). The data obtained indicate that octanoic acid is catabolized to CO_2 and water soluble products to a greater extent than palmitic acid by rat jejunal slices. Analysis of the lipid soluble products by thin layer and gas liquid chromatography revealed additional differences in the metabolism of these 2 fatty acids.

Materials and methods. 1-C^{14} octanoic and 1-C^{14} palmitic acid (New England Nuclear Corp.) were purified by thin layer chromatography. The fatty acid substrates were dissolved in a small amount of ether and homogenized in 10% crystalline bovine albumin. Non-radioactive lipid carrier was added before homogenization so that the final concentration of the lipid-albumin suspensions was 10 micromoles lipid per ml.

Aliquots of the various lipid-albumin suspensions and other water soluble products were added to 12 ml of a counting solution prepared as previously described(8). The solution prepared for counting substances in organic solvents contained 15 ml of toluene with 0.04% liquifluor (Pist Chem

Radioassay was performed in a Packard Tri-Carb liquid scintillation spectrometer. Quenching was corrected for by utilizing a channels ratio method as described by Bush(9).

Female albino rats (Charles River Breeding Laboratories) weighing 160-200 g were fasted overnight before use in these experiments. The animals were killed by a blow on the head and the small bowel rinsed *in situ* with 50 ml of ice cold 0.85% NaCl. The upper half of the intestine was everted over a glass rod and intestinal slices prepared as previously described(8). Six jejunal slices weighing between .35-.40 g were added to each incubation flask which contained the following: 0.5 ml of 5-fold concentrated low calcium Kreb's-Ringer bicarbonate buffer, pH 7.4, 0.5 ml of 0.15M $NaHCO_3$, 7.5 μmoles of glucose in 1.0 ml water, and 1.0 ml of a lipid-albumin suspension containing either 1-C^{14} octanoic or 1-C^{14} palmitic acid. The flasks were gassed for 30 minutes with 95% O_2, 5% CO_2 prior to addition of the jejunal slices and lipid substrates. In each experiment control flasks were used in which 0.25 ml of 10N H_2SO_4 was added at the start of incubation. The incubations were carried out in stoppered flasks at 37°C for 30 minutes and were terminated either by addition of 0.25 ml of 10N H_2SO_4 or placement in ice.

To measure the conversion of 1-C^{14} octanoic and 1-C^{14} palmitic acid to $C^{14}O_2$, 0.25 ml of 10N H_2SO_4 was added to stoppered flasks that were cooled in ice for 15 minutes after which 1.0 ml of hyamine 10-X was added to the center wall. The flasks remained in ice for 30 minutes after which the hyamine was removed and aliquots assayed for radioactivity.

When lipid soluble products were analyzed, the reactions were terminated by placing the flasks in ice. The tissue slices were removed, rinsed in distilled water, and blotted dry. The tissue slices and media were extracted separately by the method of Folch *et al*(10). In experiments with palmitic acid as radioactive substrate, the solvents containing the extracted lipids were evaporated to dryness under a nitrogen stream at room temperature and the residues redissolved in chloroform. In roform-methanol added at the end of the incubation contained 42 μmoles of carrier octanoic acid and 6 μmoles of carrier trioctanoin and the pH during the Folch extraction was adjusted to below 3. Because of the volatility of octanoic acid, the solvents containing the lipid extracts were evaporated slowly under a nitrogen stream in an ice bath. The dried extracts were then resuspended in chloroform. Aliquots of the lipid soluble products were assayed for radioactivity and also analyzed by thin layer and gas liquid chromatography.

Gas liquid chromatography analyses were performed using a Barber-Coleman model 25C equipped with an Argon ionization detector. The gas stream was split so that 25% was directed to the mass detector and 75% to the radioactive counter (fraction collector). The inlet pressure was 30 p.s.i. and the flow rate 80 ml per minute. Radioassay was performed by the method of Karmen *et al* (11) utilizing a Packard liquid scintillation spectrometer. For each analysis 5 μg of material in 5 μL was injected into the column. The column was coiled, 6 ft by 1/8 inch in diameter. The liquid phase was either 15% apiezon L or 20% ethylene glycol succinate and the solid support 80-90 mesh anakron. The column temperature was 197°C, the flash heat temperature 285°C, and the cell temperature 200°C.

Results. The data shown in Table I and Fig. 1 indicate that small intestinal slices *in vitro* metabolize 1-C^{14}-octanoic acid different-

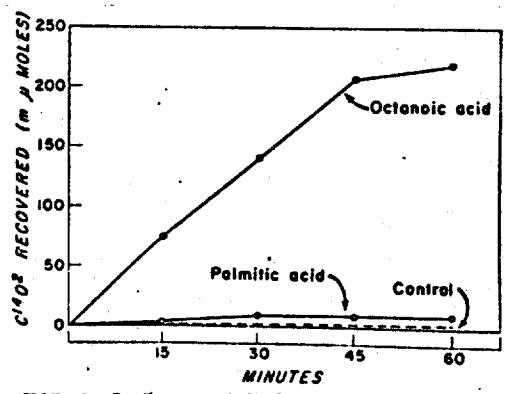

FIG. 1. Recovery of $C^{14}O_2$ after incubation of 1-C^{14} octanoic and 1-C^{14} palmitic acid with jejunal slices. Incubations were carried out for 15, 30, 45 + 60 min under conditions as described in Methods.

TABLE I. Metabolism of 1-C^{14} Octanoic and 1-C^{14} Palmitic Acid to $C^{14}O_2$, Water Soluble, and Lipid Soluble Products by Bowel Slices.

Lipid substrate (10 μmoles)	No. experiments	Distribution of recovered radioactivity (%)			Total recovery of administered radioactivity (%)
		$C^{14}O_2$	Water soluble products	Lipid soluble products	
Octanoic acid	4	1.66 ± .12 *	2.09 ± .8	96.25 ± .8	84–98
Palmitic "	4	.097 ± .003	.106 ± .009	99.80 ± .02	92–98
p value		<.01	<.01	<.01	

The component of incubation mixtures and conditions of incubation were as described in *Methods*.
* Mean ± 1 S.D.

ly than 1-C^{14}-palmitic acid. A significantly greater amount of octanoic acid was oxidized to CO_2 and water soluble products and less was recovered as lipid soluble products compared to palmitic acid. The lipid soluble products from these studies were also analyzed by thin layer and gas liquid chromatography (Tables II and III) and additional differences in the metabolism of octanoic and palmitic acids by jejunal slices demonstrated. The data in Table II indicate that a significantly greater amount of the administered palmitic acid was esterified to triglyceride as compared to octanoic acid (33% *vs* 14%; p = <.01). Furthermore, there was also a greater incorporation of palmitic acid into total neutral lipid compared to octanoic acid (41% *vs* 19%; p = <.01).

The data in Table III indicate that after incubation of C^{14}-palmitic acid with jejunal slices 96% of the label was recovered in the form of palmitic acid as determined by gas liquid chromatography. In contrast, after incubation of C^{14}-octanoic acid with jejunal slices, a small but significant fraction of the label was recovered in fatty acids other than octanoic acid. As indicated in Table III, 3.7% was recovered as decanoic acid and 4.9% was incorporated into fatty acids with chain lengths of 12-20 carbon atoms.

Discussion. A considerable amount of evidence has accumulated indicating that medium chain and long chain fatty acids are metabolized differently by the intestinal mucosa, and this information has recently been summarized(12). It has been demonstrated that a medium chain fatty acid such as octanoic is poorly incorporated into mucosal triglycerides as compared to long chain fatty acids such as myristic, palmitic, and stearic(4-7). This has been shown to be due to several factors: (1) medium chain fatty acids are poorly activated to CoA thiolesters(13,14); (2) medium chain monoglycerides are rapidly hydrolyzed by a mucosal monoester hydrolase(15,16); and (3) medium chain triglycerides may also be hydrolyzed by a mucosal lipase system(7). As a result of the low activity of mucosal esterifying enzymes towards medium chain fatty acids and the presence of an active mucosal hydrolytic system directed towards medium chain monoglycerides and triglycerides, medium chain fatty acids accumulate in the intestinal mucosa predominantly in the form of non-esterified fatty acids regardless of whether medium chain fatty acids *per se* or medium chain triglycerides are administered. In contrast, long chain

TABLE II. Thin Layer Chromatography Analysis of Lipid Soluble Products After Incubation of 1-C^{14} Octanoic and 1-C^{14} Palmitic Acid with Jejunal Slices.

C^{14}-lipid substrate (10 μmoles)	No. experiments	Analysis of	Lipid recovered (μmoles)	Distribution of radioactivity (%)				
				Monoglyceride & phospholipids	Diglyceride	Fatty acid	Triglyceride	Neutral lipid
Octanoic acid	4	Medium	7.836 ± .18*	.2 ± .17	.2 ± .1	99.4 ± .2	.2 ± .1	.6 ± .1
	4	Tissue	.806 ± .09	2.7 ± 1.50	2.6 ± 1.5	80.6 ± 8.5	14.0 ± 5.9	19.4 ± 8.5
Palmitic acid	5	Medium	8.970 ± .2	.4 ± .1	1.2 ± .2	94.6 ± 1.4	3.8 ± 1.3	5.4 ± 1.3
	5	Tissue	.905 ± .08	4.4 ± .3	3.8 ± .5	58.8 ± 7.2	33.0 ± 7.5	41.2 ± 7.5

* Mean ± 1 S.D.

TABLE III. Gas Liquid Chromatography Analysis of Lipid Soluble Products Obtained After Incubation of Jejunal Slices with Either 1-C^{14} Octanoic Acid or 1-C^{14} Palmitic Acid.

Lipid substrate	Analysis of	No. experiments	Distribution of radioactive label in fatty acids (%)										Recovered radioactivity, dpm × 10^{-3}
			8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:0	
1. Octanoic acid	Medium	2	99.0										9.2
	Tissue lipids	3	91.1	3.7				4.9					2.8
2. Palmitic acid	Medium	2					99.0		2.0	<1.0	<1.0	<1.0	8.0
	Tissue lipids	4			<1.0		93.6	<1.0					

fatty acids are present in intestinal mucosa primarily in the form of triglycerides after administration of either long chain fatty acids or long chain triglycerides(4-7).

The data obtained in the present study indicate that a significantly greater amount of palmitic acid was esterified to triglyceride and neutral lipids by intestinal mucosa as compared to octanoic acid. However, it is noteworthy that after incubation of 10 μmoles of octanoic acid with jejunal slices, 0.156 μmoles were recovered as neutral lipid (Table II). This value is somewhat higher than has been reported by others(4,5) and could possibly be related to the following factors: (1) tissue slices were used which may be more active than tissue homogenates in the esterification of fatty acid substrate(4); (2) larger amounts of lipid substrate (10 μmoles) were utilized in comparison with previous studies; and (3) cold carrier lipid was used to minimize loss of radioactivity during the extraction process.

It was of interest that octanoic acid was catabolized by intestinal mucosa to a greater extent than palmitic acid. This was also reflected by the observation that 9% of the C^{14} label from octanoic acid was incorporated into fatty acids with chain lengths of 10-20 carbon atoms (Table III). This suggests that some of the octanoic acid may first have been catabolized to 2 carbon fragments (*i.e.*, acetate) which were subsequently incorporated into long chain fatty acids. Since medium chain fatty acids are poorly activated to CoA thiolesters(13,14), it seems unlikely that the octanoic acid underwent elongation reactions to yield the longer chain fatty acids.

Summary. Measurements were carried out on the *in vitro* metabolism of C^{14}-octanoic and C^{14}-palmitic acid by rat intestinal slices. Significantly more octanoic acid was catabolized to CO_2 and water soluble products and less recovered as lipid soluble products compared to when palmitic acid was the substrate. Analysis of these lipid soluble products by thin layer chromatography revealed that more of the palmitic acid was esterified to triglyceride compared to octanoic acid. Further analysis of the lipid soluble products by gas liquid chromatography demonstrated that a small but significant amount (9%) of the label from C^{14}-octanoic acid was recovered in fatty acids with chain lengths of 10-20 carbon atoms. This suggests that some of the C^{14}-octanoic acid may have been catabolized to C^{14}-acetate which was then incorporated into long chain fatty acids. In contrast, virtually all of the label from C^{14}-palmitic acid was recovered as such, indicating that very little mucosal catabolism of palmitic acid had occurred.

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The Presence of Lymphocytes in Long Term Cultures of Newborn Mouse Thymic Epithelium.* (30566)

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Observations of *in vitro* cultures of newborn thymus fragments followed for as long as 120 days have been reported(1). We will report here the observations on cultures derived from newborn mouse thymus fragments initially explanted in a plasma clot and followed for over 22 months. The term epithelial cell, instead of that of reticulum cell employed in our previous work, will be used in the present note to describe the cells originating from the epithelial reticulum of the thymus. This appears justified both by the morphological appearance of the cells observed in culture and by the embryological derivation of the thymus(2,3,4,5,6,7).

Material and methods. Source of the thymus was a 24-hour-old MA mouse. The mouse was killed with ether and the thymus aseptically removed to a Petri dish containing Hanks' BSS. After washing, the thymus was transferred to a second Petri dish containing Hanks' BSS and finely minced. The minute fragments were then transferred to 2 Leighton tubes (Bellco Glass Co., Vineland, N. J.) into which a coverslip had been previously inserted. Two drops of chicken plasma (Difco) were pipetted to the coverslip before inserting the thymus fragments into the tube. The plasma solidified rapidly after transfer of the fragments and the medium was then added. This procedure favored the adhesion of the fragments to the glass.

The culture medium was Hanks' BSS with phenol red as indicator, plus vitamins, glutamin, essential amino acids and was supplemented with 11% fetal bovine serum (Microbiological Associates, Bethesda, Md.). No antibiotics were used. The medium was first changed after 4 days and thereafter changed every 5-7 days. Every 2 changes, after the old medium was discarded, the culture was washed with Hanks' BSS before the fresh medium was added.

Of the original cultures, one was sacrificed

FIG. 1. 4-day-old culture. Macrophages phagocytizing lymphocytes and debris, surrounded by dead or degenerating lymphocytes. 250 ×.
FIG. 2. 14-day-old culture, large lymphocytes growing on background of a continuous sheet of epithelial cells. Original magnification 250 ×, enlarged 3 times. Phase contrast.
FIG. 3. 14-day-old culture. Lymphocytes growing on epithelial cells. May-Grünwald and Giemsa, 250 ×.
FIG. 4. 15-month-old culture. Mitosis of an epithelial cell. Original magnification 250 ×, enlarged 3 times. Phase contrast.
FIG. 5. 22-hour-old subculture from the 14-month-old original culture. Colony of epithelial cells. May-Grünwald and Giemsa, 110 ×.
FIG. 6. 18-hour-old subculture from the 14-month-old original culture. Small colony of epithelial cells. A few lymphocytes with pyknotic nuclei are visible. One is engulfed in a cytoplasmatic vacuole. May-Grünwald and Giemsa, 250 ×.

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FATTY ACID METABOLISM OF RAT DIAPHRAGM*

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Recent studies (1, 2) have indicated that hepatectomized animals have the ability to oxidize fatty acids directly to carbon dioxide. We have obtained evidence which indicates that rat diaphragm oxidizes exogenous octanoic acid *in vitro*. Inasmuch as the feeding of diets high in fat has been found to limit subsequent utilization of glucose by the diaphragm (3), the effect of diet on this oxidation of octanoate has been studied.

EXPERIMENTAL

All the animals used were of a Sprague-Dawley strain. Stock animals were maintained on a standard Purina fox chow diet. In the cases in which the caloric intake was principally fat or carbohydrate, however, the diets were identical with those used in a previous study (3). The animals were sacrificed by decapitation, and their diaphragms removed and incubated under an atmosphere of oxygen in Krebs-Ringer phosphate buffer at pH 7.4 with other additions as indicated. The octanoic acid used was redistilled from a sample obtained from the Eastman Kodak Company. The neutral equivalent of the acid and the melting point of its amide derivative agreed with the theoretical values. Octanoic acid was determined by the method of Lehninger and Smith (4).

Octanoate Uptake by Diaphragm from Animals Fed High Carbohydrate and High Fat Diets—The hemidiaphragms from animals in each group were paired between a control flask and a flask containing octanoate in the side arm. After equilibration, octanoate was added to the main compartment, and incubation continued for 2 hours. The final octanoate concentration in the medium was determined and used to calculate the octanoate uptake per 100 mg. of fresh tissue. In all the experiments, at the 2.0 and 3.3 μM level of substrate (Table I), the carbohydrate tissues removed more octanoate from the medium than did the fat tissues. Octanoic acid significantly increased oxygen consumption of all tissues during the period observed.

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Octanoate Oxidation by Diaphragm from Animals Fed Stock Diet—Stock animals weighing from 150 to 250 gm. were sacrificed. The diaphragm was removed; one hemidiaphragm was taken as a control, and the other incubated in the presence of octanoate. The octanoic acid was added from the side arm to give a final concentration from 0.5 to 5.0 μM per ml. of medium. Oxygen consumption is expressed as micromoles of oxygen removed from the medium per 100 mg. of tissue for the first and the second parts of the 120 minute incubating period. The presence of substrate increased the oxygen consumption in all experiments except one for the first 60 minute period (Table

TABLE I

Uptake of Octanoic Acid and Oxygen by Diaphragm from Rats Fed High Fat and High Carbohydrate Diets

Each experiment includes hemidiaphragms from three animals for each dietary group. The tissues from each rat were paired between a control flask and a flask containing octanoate in the side arm. After equilibration and addition of substrate, the tissues were incubated 120 minutes as usual. The oxygen consumption is reported for the first 60 minute period only. The results are expressed as micromoles per 100 mg. of wet tissue.

Experiment No.	Octanoate concentration	Oxygen uptake				Octanoate uptake	
		Carbohydrate diet		Fat diet		Carbohydrate diet	Fat diet
		Control	Octanoate	Control	Octanoate		
	μM per ml.						
1	2					0.9	0.8
2	2					1.2	0.6
3	2	6.3	7.0	5.8	6.3	1.0	0.8
4	2	6.2	7.0	5.8	6.4	1.0	0.8
5	2	7.0	7.9	6.1	7.7	1.4	1.0
6	3.3					1.3	0.8
7	3.3	6.8	8.4	6.6	7.3	1.4	1.1
8	3.3	6.3	6.5	5.8	6.7	0.9	0.8
9	3.3	7.0	8.8	6.1	7.7	1.5	1.3
10	3.3	5.5	6.7	5.8	6.0	1.4	1.0
11	6.3	5.6	6.8	4.9	5.8	1.4	1.3
12	6.3	6.8	7.3	6.6	7.4	1.9	1.2
13	6.3	5.5	6.5	5.8	7.1	1.5	2.0
Average ± s.e.m.*.....		6.3 ±0.19	7.3 ±0.25	5.9 ±0.15	6.8 ±0.07	1.3 ±0.08	1.0 ±0.10
Student's *t*.....		5.93†		5.93†		3.06†	

* Standard error of mean.
† Highly significant difference.

T

Effect of Octanoic Acid on

The tissues from stock rats were pai phate buffer and a flask containing bu pressed as micromoles per 100 mg. of w

Octanoate concentration	First 6 Oxygen u Control
0.5	5.0
0.5	6.6
1.0	6.5
1.0	7.4
3.3	5.2
3.3	5.1
3.3	5.2
3.3	5.2
3.3	6.0
3.3	5.8
3.3	6.2
3.3	5.6
3.3	5.9
4.0	6.0
4.0	6.1
4.0	6.3
5.0	5.2
Average ± s.e.m.....	5.8 ±0.16
Student's *t*.....	6.12*

* Highly significant difference.

Time Course of Octanoate Uptake i from six stock rats were removed as into three approximately equal portic distributed in six flasks. One flask were equilibrated. Sodium octanoat ive a concentration of 4.0 μM per n indicated, the flasks were removed,

n from Animals Fed Stock Diet—Stock gm. were sacrificed. The diaphragm was taken as a control, and the other

ABLE I

Diaphragm from Rats Fed High Fat and High ydrate Diets

ragms from three animals for each dietary paired between a control flask and a flask after equilibration and addition of substrate, as usual. The oxygen consumption is re- y. The results are expressed as micromoles

Oxygen uptake			Octanoate uptake	
Fat diet			Carbohydrate diet	Fat diet
tanoate	Control	Octanoate		
			0.9	0.8
			1.2	0.6
7.0	5.8	6.3	1.0	0.8
7.0	5.8	6.4	1.0	0.8
7.9	6.1	7.7	1.4	1.0
			1.3	0.8
8.4	6.6	7.3	1.4	1.1
6.5	5.8	6.7	0.9	0.8
8.8	6.1	7.7	1.5	1.3
6.7	5.8	6.0	1.4	1.0
6.8	4.9	5.8	1.4	1.3
7.3	6.6	7.4	1.9	1.2
6.5	5.8	7.1	1.5	2.0
7.3 ±0.25	5.9 ±0.15	6.8 ±0.07	1.3 ±0.08	1.0 ±0.10
	5.93†		3.06†	

ioate. The octanoic acid was added ncentration from 0.5 to 5.0 μM per ml. s expressed as micromoles of oxygen ng issue for the first and the sec- bating period. The presence of sub- ption in all experiments except one for

the first 60 minute period (Table II). At 0.5 and 1.0 μM levels of octanoate, this stimulation continued throughout the incubation period. Higher levels of octanoate usually inhibited oxygen consumption during the latter phase of the incubation.

TABLE II

Effect of Octanoic Acid on Oxygen Uptake of Rat Diaphragm

The tissues from stock rats were paired between a control flask containing phosphate buffer and a flask containing buffer plus octanoic acid. The results are expressed as micromoles per 100 mg. of wet tissue.

Octanoate concentration	First 60 min.		Second 60 min.		
	Oxygen uptake		Oxygen uptake		Per cent changes
	Control	Octanoate	Control	Octanoate	
0.5	5.0	6.5	4.4	5.3	+20
0.5	6.6	7.5	6.2	6.9	+11
1.0	6.5	8.6	6.2	7.7	+24
1.0	7.4	9.0	6.7	8.5	+27
3.3	5.2	6.0	4.8	5.4	+13
3.3	5.1	7.3	4.9	5.6	+14
3.3	5.2	5.5	4.9	4.4	−10
3.3	5.2	6.2	4.9	3.2	−32
3.3	6.0	6.7	6.1	4.2	−31
3.3	5.8	7.2	5.2	4.8	−8
3.3	6.2	5.9	5.7	4.3	−25
3.3	5.6	7.2	5.3	4.4	−17
3.3	5.9	6.4	5.6	5.4	−4
4.0	6.0	6.3	6.6	5.3	−20
4.0	6.1	6.7	6.4	5.5	−14
4.0	6.3	6.9	6.6	5.7	−14
5.0	5.2	6.3	4.2	2.7	−36
Average ± s.e.m.....	5.8 ±0.16	6.8 ±0.22			
Student's *t*.....	6.12*				

* Highly significant difference.

Time Course of Octanoate Uptake by Rat Diaphragm—The diaphragms from six stock rats were removed as usual; each hemidiaphragm was cut into three approximately equal portions, and the resulting segments were distributed in six flasks. One flask was boiled for 2 minutes, then all were equilibrated. Sodium octanoate was added from the side arm to give a concentration of 4.0 μM per ml. of medium, and, at the intervals indicated, the flasks were removed, and the remaining octanoic acid

was determined. There was an initial period of rapid removal of octanoate from the medium (Fig. 1). This rate decreased markedly with time. Although no oxygen was consumed by the boiled tissue, it appears that

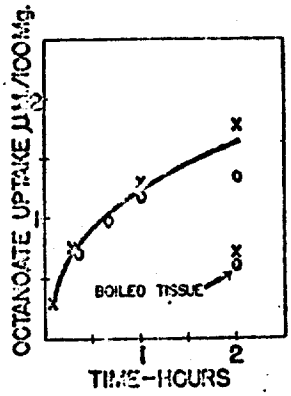

FIG. 1. Stock rats were used in this study. The hemidiaphragms from six animals were cut into three equal portions each and the resulting segments were distributed in six flasks. Octanoic acid was added from the side arm to give a concentration of 4.0 μM per ml. of medium. At the intervals indicated, the flasks were removed and the remaining octanoate was determined. The results of two similar experiments are given.

TABLE III

Effect of Acetate on Oxygen Consumption and Glycogen Formation of Rat Diaphragm

The tissues from stock rats were paired between a control flask containing glucose in phosphate buffer at a level of 0.05 per cent and a flask containing the same concentration of glucose plus 0.1 per cent acetate (17.2 μM per ml.) in a phosphate buffer. The results are expressed as micromoles per 100 mg. of wet tissue.

Experiment No.	Oxygen consumption			
	First 60 min. period		Second 60 min. period	
	Control	Acetate	Control	Acetate
1	7.0	8.6	5.7	7.7
2	7.0	7.7	6.0	6.7
3	7.3	7.9	6.0	7.1
4	6.4	7.3	5.4	6.9
5	6.3	7.6	5.4	7.6
6	6.3	7.0	5.4	6.9
7	6.7	8.4	5.7	8.0
Average ± s.e.m.....	6.7 ±0.15	7.8 ±0.16	5.7 ±0.1	7.3 ±0.19
Student's *t*.....	6.22*		7.21*	

* Highly significant difference.

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Effect of Acetate on Rat Diaphra
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DISC

Data, presented by Parnes and
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sumption reported here give infere
acetate. A more detailed study of
glycogen change is in progress.

The decreased octanoate uptake
the fat diet is difficult to understand
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SUMM

1. Octanoic acid in relatively lo
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3 μM per ml. of medium, however, an
stimulation.

2. Acetate at a level of 17.2 μM per
consumption of the isolated rat diap

3. The isolated diaphragms from

approximately 0.7 μM of octanoate was bound. The tissue itself, therefore, appears to have an appreciable affinity for octanoate.

Effect of Acetate on Rat Diaphragm—In view of the stimulation in oxygen consumption of diaphragm by octanoate, which was observed in this study, and an inhibition of oxygen consumption by acetate, reported by Parnes and Wertheimer (5), an attempt was made to duplicate their experiment. Glucose and acetate were added to the medium at the levels indicated by Parnes and Wertheimer, and oxygen consumption was measured. In all the experiments (Table III), acetate increased the oxygen consumption of diaphragm throughout the incubation period. This stimulation is quite similar to that observed with low concentrations of octanoate.

DISCUSSION

Data, presented by Parnes and Wertheimer (5) without comment, indicated that the oxygen consumption of rat diaphragm was inhibited by acetate. These data are in direct contrast to the findings in this laboratory. It should be noted that the effects of octanoate on oxygen consumption reported here give inferential support to our experiments with acetate. A more detailed study of the effect of acetate and octanoate on glycogen change is in progress.

The decreased octanoate uptake by the tissues from the animals fed the fat diet is difficult to understand except in terms of a physicochemical effect. Octanoate is bound by boiled tissue and could be bound even more vigorously by surviving diaphragm. If protein plays a part in this binding, the presence of comparatively large amounts of lipide could limit further binding. Of course, these results could also reflect an enzymatic difference.

The inhibition of oxygen consumption with high concentrations of octanoate in the latter phases of the incubation may be related to the well known detergent properties of this type of molecule. The inhibition becomes apparent about midway in the incubation period. This time-uptake curve demonstrates that the removal of octanoate from the medium is approaching a maximum at that time.

SUMMARY

1. Octanoic acid in relatively low concentrations stimulates oxygen consumption by rat diaphragm. When the original concentration exceeds 3 μM per ml. of medium, however, an inhibition usually follows the initial stimulation.

2. Acetate at a level of 17.2 μM per ml. of medium stimulates the oxygen consumption of the isolated rat diaphragm.

3. The isolated diaphragms from animals, previously maintained on a

high carbohydrate intake, remove more octanoate from the medium tha do similar tissues from animals fed a fat diet. Even so, tissues fro both groups of animals consumed more oxygen when octanoate w present.

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A NEPHELOMETRIC DETERMINATION

By R. G. HANSEN, W. J. RUT

(From the Laboratory of Chemistry, Depar Illinois, Urban

(Received for publication,

The most widely accepted procedure pears to be that of Good, Kramer, an inciples used by Bernard (2). The t queous potassium hydroxide. Advant lity of glycogen in aqueous alcohol to s the digest and to bring about further ns. The sample is finally hydrolyzed ted from the reducing power of the s This paper reports a new procedure for incipal advantage of which is that a dec required. The turbidity of suspensi hol is the basis of the method suggested i

Solutions—

1. Potassium hydroxide; 30 gm. of (a ved in 100 ml. of distilled water.
2. Sodium chloride; 2 per cent aqueous.
3. Ethanol; 95 per cent.
4. Dreft; 1 per cent aqueous.
5. Precipitation solution; 1 part of 2 per cent ethanol.
6. Alcohol reagent; 210 ml. of 95 per cent Cl, 30 ml. of 1 per cent Dreft, and 30 ml.

Procedure

Tissue containing from 0 to 2 mg. of glycog sing in a centrifuge tube in 30 per cent utes in a boiling water bath. For rat dia of tissue are placed in 0.5 ml. of base. of the mixture of 1 part of 2 per cent sodi er cent ethanol. The contents of the tu um of air through a capillary. The tube ath until bubbles are observed in the diges rator for at least 3 hours to allow complete

Experientia 13:331-3 (1957).

Table II

Brain and large intestine 5 HT concentrations (biological method).

Treatment	Time after treatment	Number of animals	Number of determinations	Encephalic Serotonine mg/g ± S.E.	Range	Intestinal Serotonine mg/g ± S.E.	Range
. . –	–	27	9	13 ± 4	6– 48	2300 ± 300	1200-3600
Electroshock . . .	10 min	27	9	360 ± 7	90-720	920 ± 100	480-1400
Electroshock . . .	1 h	22	7	160 ± 40	48-300	1530 ± 300	960-3000
Electroshock . . .	2 h	22	7	47 ± 10	12– 72	1780 ± 470	1080-3600
Electroshock . . .	24 h	22	7	13 ± 3	4– 36	2370 ± 300	1740-3600

Same assays were carried out on the test of rat's isolated uterus. Also in this test, 10 min after electroshock, an increase in cerebral serotonin can be observed. Also after cardiazol (80 mg/kg intraperitoneally), the variations in encephalic and intestinal serotonin are similar.

We also wish to point out the difference between our results and those obtained after reserpine administration, when a fall in brain 5 HT concentration (and not a marked raise, as in our experiments) parallels the fall of intestine 5 HT concentration.

Addendum: Further determinations, with spectrophotometric method, show after electroshock a second increase in brain 5 HT starting from the 36th hour to the 72nd hour. In the same time intestinal levels of 5 HT do not change.

S. Garattini, A. Valsecchi, and L. Valzelli

Institute of Pharmacology, University of Milano (Italy), March 11, 1957.

Riassunto

Si dimostra che dopo uno shock convulsivo elettrico o cardiazolico il contenuto in serotonina aumenta in maniera molto rilevante nell'encefalo e si abbassa nell'intestino crasso.

L'aumento encefalico e la diminuzione intestinale, che raggiungono gradi molto cospicui già 10 min dopo il trattamento convulsivante si attenuano poi nei tempi successivi: intensamente diminuiti dopo 1 h, non sono più rilevabili dopo 24 h.

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B. Holmquist

Effects of Short Chain Fatty Acid Anions upon Cortical Blood Flow and EEG in Cats

It has recently been reported by White and Samson and by Samson and Dahl[2] that buffered fatty acid anion solutions, injected intravenously in rabbits, produce slow waves in the EEG in combination with behavioural signs of sleep. Furthermore, it has been demonstrated that fatty acids to some extent may participate in the normal cerebral metabolism in man[3].

Investigating the influence of various metabolites upon the EEG and the cortical blood flow in the cat recorded with a new method[4], we have injected small doses of butyric (1 *M*) and octanoic (0·5 *M*) acid solutions (buffered with NaOH) as well as a solution of oleic acid (dissolved in 10% human albumine)[5]. Heparinized preparations were used, either lightly anaesthetized (Nembutal) or unanaesthetized (*encéphale* or *cerveau isolé*). The EEG, the blood pressure and the cortical blood flow were recorded continuously during the experiments.

No effects were obtained by the oleic acid solution. Octanoic anions had the same effect as butyric anions.

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[5] The solutions were kindly prepared by B. Borgström, M. D., Department of Biochemistry, University of Lund.

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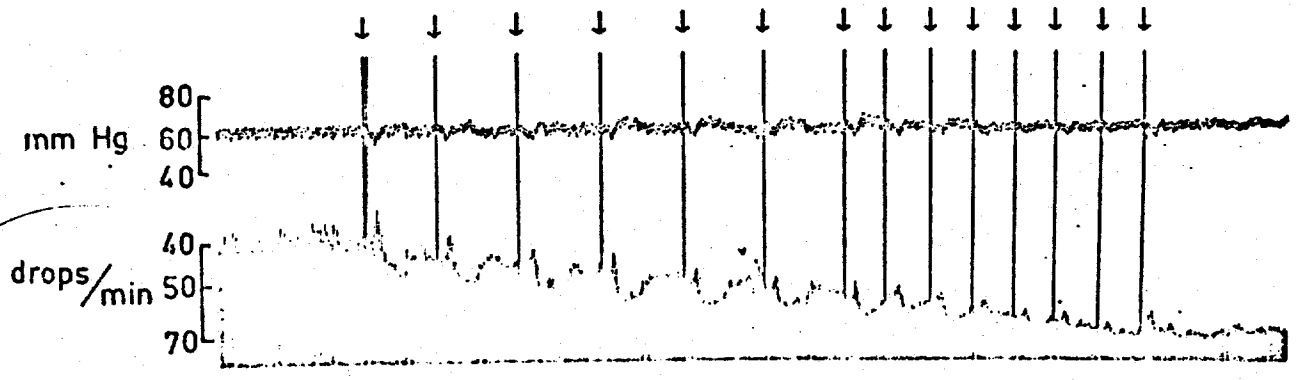

Fig. 1. – Cat. *Cerveau isolé* preparation. Cervical sympathetic nerves sectioned. Records of *blood pressure* (recorded by damped electromanometer in the brachial artery) and *cortical blood flow* in rostral parts of the brain. The flow record consists of dense vertical lines. The height of each line is proportional to the interval between blood drops from the cannulated superior sagittal sinus. Decreasing height of vertical lines therefore means increasing flow. Record interrupted every 30 s. Arrows indicate intravenous injections of 1 ml of 1 *M* buffered butyric acid solution. Note increase of cortical blood flow in spite of constant blood pressure.

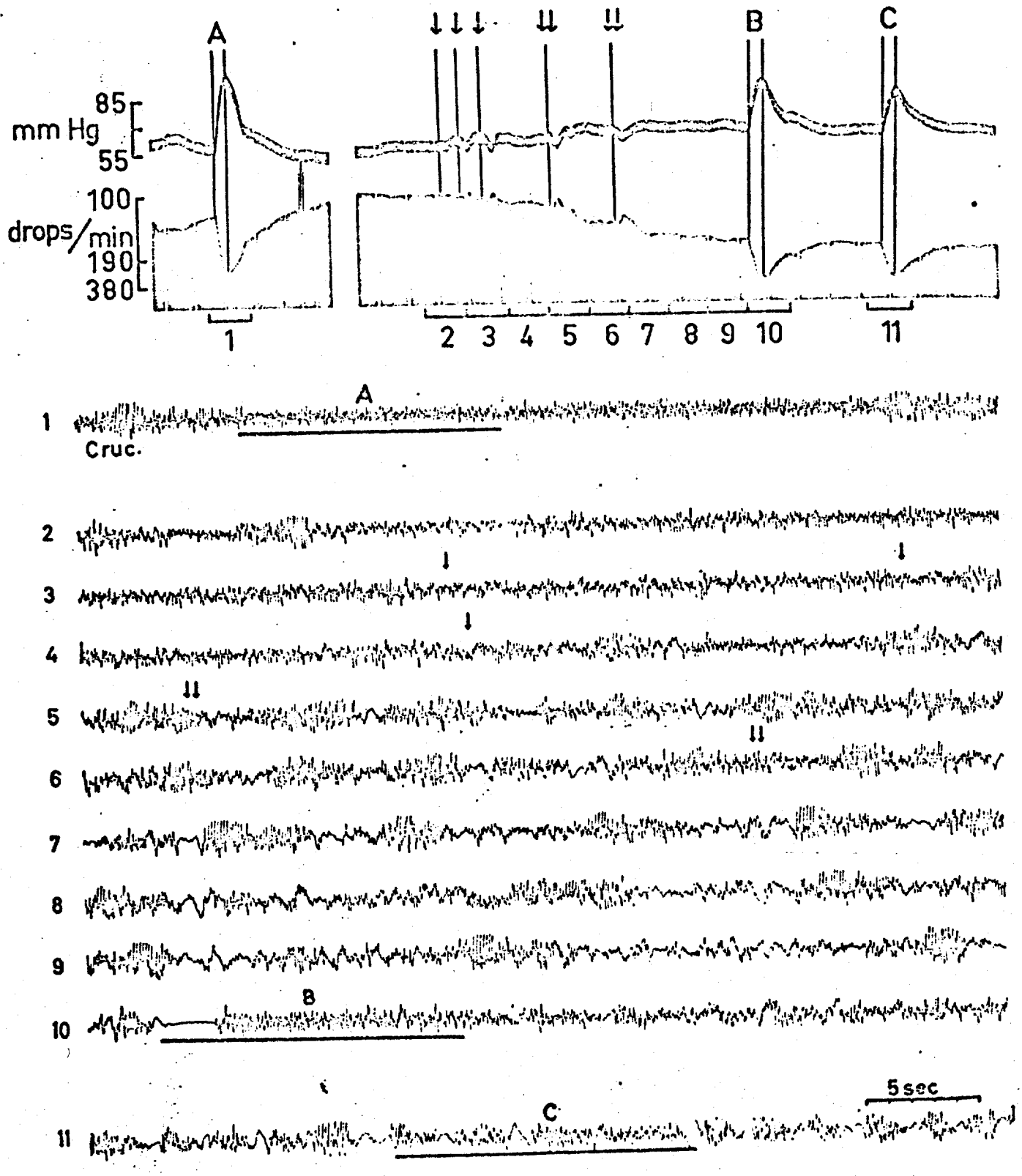

Fig. 2.—Cat. Light Nembutal anaesthesia. Vagi and cervical sympathetic nerves sectioned. *Upper diagram:* Records of *blood pressure* and *cortical blood flow* as in Figure 1. *Lower diagram:* Samples of *EEG* from cruciate area. Numbers correspond to periods marked below upper diagram. Calibration 200 microvolts. *A*, *B* and *C* electrical stimulation of the mesencephalic reticular formation (threshold intensity, 250/s, 1 ms duration). Each arrow indicates injection of 1 ml of 1 *M* buffered solution of butyric acid. Note: *1* Slow waves in the EEG started to develop after third dose of butyric acid. *2* Increase of cortical blood flow evident from period 6 and on. *3* The brain stem stimulation which at *A* gives a typical arousal reaction of the EEG becomes less effective after butyric acid in *B* and is without effect in *C*. The pressor responses are also diminished in *B* and C as compared to *A*.

Both were found to produce the change of EEG previously described[6]. It was furthermore observed that at the same time as developed of slow waves and spindles in the EEG, there was a pronounced increase of the cortical blood flow due to a reduction of the cortical vascular resistance (Fig. 1). A relative increase of flow of up to 100% was thus recorded after administration of 4 *mM*/kg of butyric acid. Minor effects upon the EEG and the blood flow were seen already after injection of 1/2 ml of the solution used. The presence of glucose in large concentrations in the blood did not alter the effects described.

When arousing stimuli (adequate stimulation, electrical stimulation of the brain stem, injection of Metrazol) were employed as a test of brain stem functions, it was found that the arousal reaction of the EEG disappeared completely with increasing doses of the fatty acid solution (Fig. 2). Pressor effects following brain stem stimulation also diminished. These facts demonstrate that the fatty acid anions block the general activating influence of the reticular system upon the cortex[7].

The finding of synchrony in the EEG with slow waves in combination with cortical vasodilatation following injection of short chain fatty acids is of interest for several reasons. This state contrasts with the one obtained with e.g. barbiturates, which also induce synchrony but with some cortical vasoconstriction[8]. On the other hand, the fatty acid effects show principal similarities with the state of physiological sleep in which there is a sleep pattern in the EEG as well as some cortical vasodilatation[9]. It is known that local metabolites are of great importance for the adjustment of the cortical vasomotor tone during changes in EEG[10]. In view of the findings reported here, it seems an interesting suggestion for further investigation that fatty acids as humoral agents may participate in the normal regulation of cortical excitability and vasomotor tone.

B. Holmquist and D. H. Ingvar

Institute of Physiology, University of Lund (Sweden), March 12, 1957.

Zusammenfassung

Die Wirkung von intravenös injizierten Fettsäureanionen (0,5–1,0 molare Lösung in NaOH von Buttersäure und Kapronsäure) auf das Elektroencephalogramm, auf den Blutdruck und auf die kortikale Durchblutung wurde bei leicht narkotisierten und nichtnarkotisierten Katzen untersucht. Es wurde bestätigt, dass Fettsäureanionen eine ausgesprochene Depression des Elektroencephalogramms herbeiführen. Weiterhin wurde gezeigt, dass sich mit dieser Depression auch eine bedeutende Herabsetzung des kortikalen Gefässwiderstandes entwickelt.

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Elektrophysiologische Untersuchungen über die Ultraviolettempfindlichkeit von Insektenaugen[1]

Bei der Frage nach der spektralen Absorption der Sehstoffe im Komplexauge der Insekten nehmen elektrophysiologische Messungen der spektralen Empfindlichkeit einen bevorzugten Platz ein, da wegen methodischer Schwierigkeiten direkte Bestimmungen bisher fehlen. Für das sichtbare Spektrum liegen solche Messungen bereits vor[2]. Schon ältere Verhaltensversuche haben indessen gezeigt, dass von vielen Insekten ausser dem sichtbaren auch das für uns weitgehend unsichtbare ultraviolette Licht (UV) wahrgenommen wird und zum Teil erstaunlich reizwirksam ist[3]. Da diese Versuche wenig Rückschlüsse auf die spektrale Absorption der Pigmente zulassen, lag es nahe, die spektrale Empfindlichkeit für das UV elektrophysiologisch zu bestimmen.

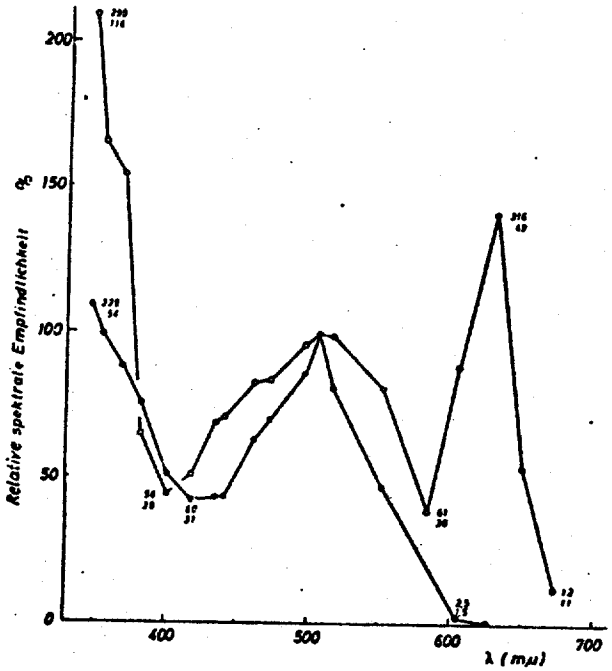

Abb. 1. Relative spektrale Empfindlichkeit von *Calliphora erythrocephala* (o) und *Periplaneta americana* (oberer Augenteil; •) bei Dunkeladaptation. Relative spektrale Empfindlichkeit: Reziprokwerte derjenigen Quantenzahlen, die als Einzelreiz Ein-Effekte gleicher Höhe erzeugen, in Prozent des Maximums bei 507 mμ. Mittel der Werte aus 7 bzw. 6 Versuchen, deren Variabilität durch die angeschriebenen Extrema demonstriert wird.

Als Lichtreizgerät diente ein Doppelinterferenzfilter-Monochromator mit einer Xenonlampe als Lichtquelle[4]. Es wurden Fliegen (*Calliphora erythrocephala*, Wildform[5]) und Schaben (*Periplaneta americana*, nur oberer Augenteil) untersucht, deren spektrale Empfindlichkeitsverteilung im für uns sichtbaren Spektrum bekannt ist[6]. Die sonstigen Versuchsbedingungen entsprechen im wesentlichen den Angaben dieser Autoren.

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Anal. Chem. 32:682-4 (1960)

Gas Chromatographic Separation of Volatile Organic Acids in Presence of Water

66

IRVING R. HUNTER, VICTOR H. ORTEGREN, and JAMES W. PENCE
Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.

▶ Volatile organic acids including acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, and caprylic acids have been separated by gas-liquid chromatography using an adipate polyester of diethylene glycol as the liquid phase and helium as the carrier gas. Fractions emerging from the column were oxidized to carbon dioxide which was then measured in thermal conductivity cells. Nearly complete resolution of most of the acids was readily obtained, even in the presence of water in amounts up to 50% of the mixture.

SEPARATION of volatile fatty acids by gas chromatography was first reported by James and Martin in 1952 (*2*). These investigators worked with anhydrous mixtures of volatile organic acids and a column in which the liquid phase was DC 550 silicone oil containing 10% of stearic acid. The eluted fractions were measured by a recording buret coupled to a photoelectric control circuit.

Until very recently this procedure, or its modifications (*1*), appears to have been the only one developed for the separation of organic acids by gas chromatography, despite the widespread use and rapid growth of this technique. Two factors which undoubtedly contributed to the lack of progress were the need for strict maintenance of anhydrous conditions and the corrosiveness of acid vapors on metallic elements of the usual types of sensitive measuring equipment.

At the 1956 Institute of Petroleum (London) symposium on vapor phase chromatography successful separations of 20% aqueous solutions of mixed organic acids were mentioned, but with no description of the equipment or techniques used (*4*). More recently Smith (*6*) described the separation of aqueous mixtures of formic, acetic, propionic, isobutyric, and *n*-butyric acids using Tween 80 (5%) on Celite 545. He also described the separation of formic, acetic, isobutyric plus propionic as a single peak, and *n*-butyric by the use of Carbowax 400 on Celite.

This paper describes a technique for the analytical separation of mixtures of organic acids up through C_8, whether anhydrous or in aqueous solution. The essential features are: the use of diethylene glycol adipate polymers and related materials as the liquid phase; glass columns; and the combustion of eluted acids by a modification of the method of Martin and Smart (*5*) prior to passage through a thermal conductivity cell. Water admixed with the organic acids does not adversely affect the separation, and the cell filaments and block are exposed only to the carbon dioxide formed by combustion of the acids. Water of combustion is removed in a drying tower ahead of the cell.

APPARATUS

The gas chromatographic apparatus (Figure 1) was constructed in the laboratory. The assembled apparatus consisted of a controlled gas source, sample inlet, four Gow-Mac filaments (two in each of the reference and sample

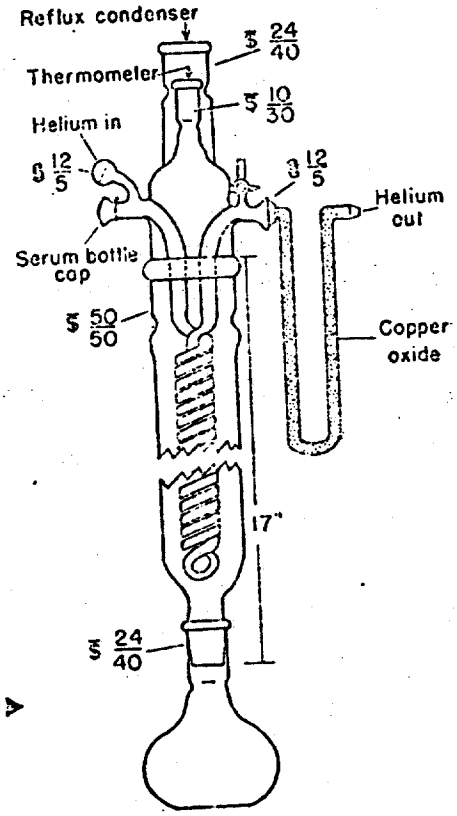

Figure 1. All-glass column and temperature control jacket for separation of volatile organic acids by gas chromatography

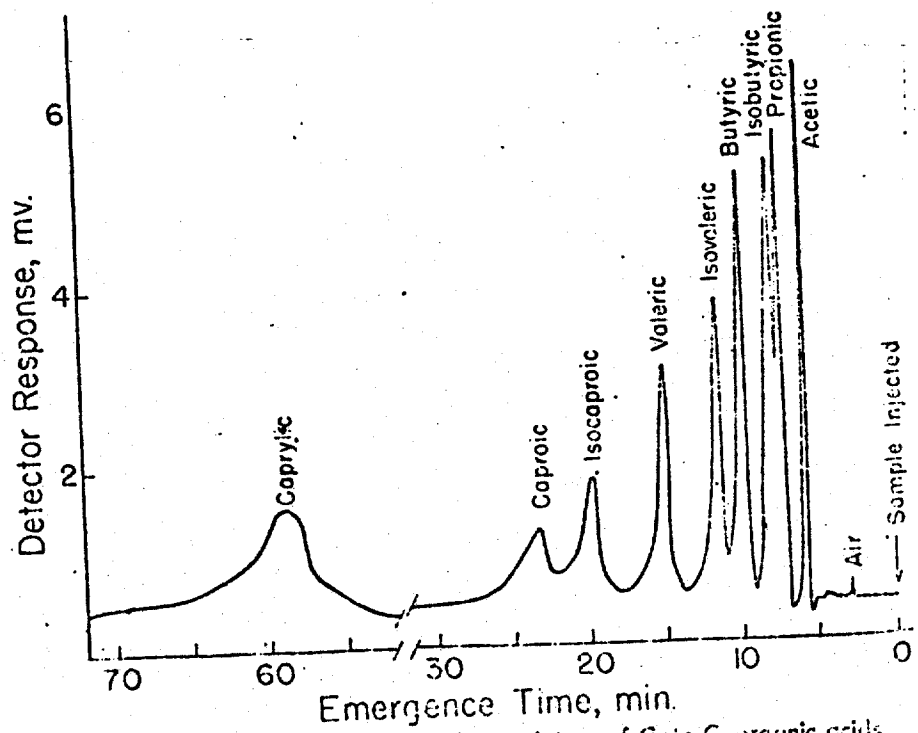

Figure 2. Chromatogram of anhydrous mixture of C_2 to C_8 organic acids

Sample size, 10 μl.

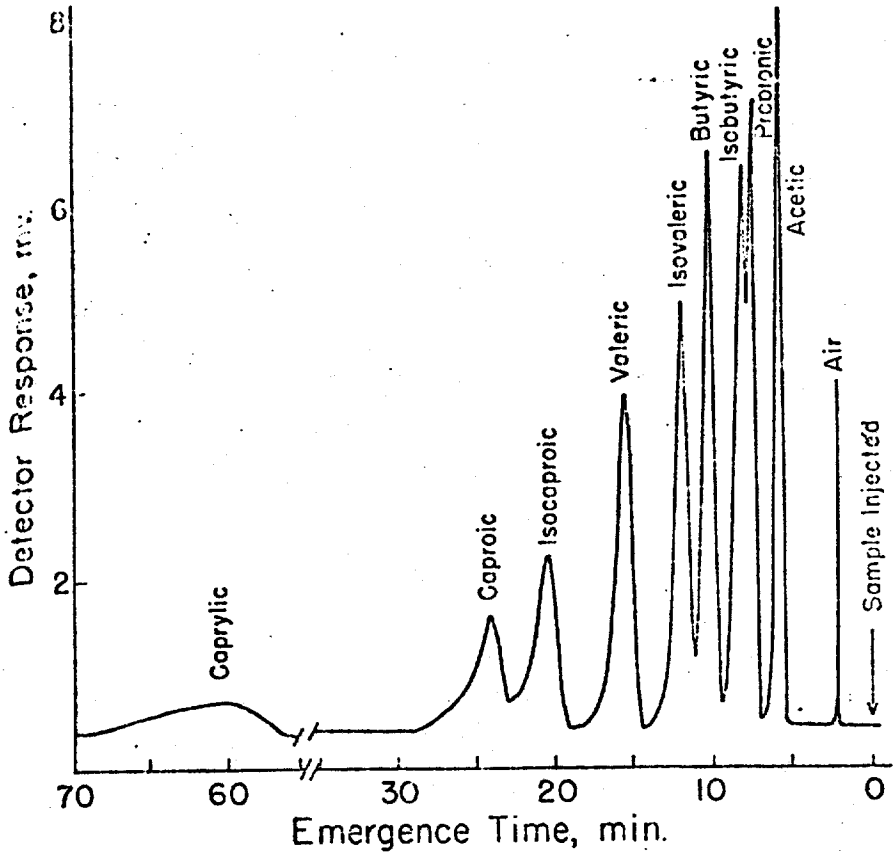

Figure 3. Chromatogram of aqueous mixture of C_2 to C_5 organic acids

Sample size, 20 μl.

detectors), column, flowmeter, vapor jacket, combustion furnace, drying agent, and the necessary circuitry for detecting, amplifying, and recording the detector signal.

Column. The chromatographic column, constructed of 8 feet of tubing 4 mm. in inside diameter, was wound in the form of a helix about 1.5 inches in diameter. The first and last 2 inches of the column were made of heavy-walled tubing (3 mm. i.d.) sealed into the top of a vapor jacket provided with a 50/50 standard taper joint. This was cut to a 15-mm. length to provide a large enough opening for the glass-blowing work.

Attached to the inlet of the column was a short side arm shaped to receive a rubber serum-bottle cap through which samples could be injected with a syringe. The outlet of the column ended in a 12/5 outer spherical joint which was heated electrically during a run by several windings of glass-covered Nichrome wire (24-gage B & S, with a resistance of 1.67 ohms per foot) to prevent condensation of acids as they emerged from the column.

The column packing was 25- to 40-mesh Johns-Manville C-22 firebrick coated with 15 grams of the adipate polyester of diethylene glycol per 100 grams of brick. This column packing was prepared by dissolving the ester in chloroform and then mixing in the firebrick. The solvent was then evaporated under vacuum in a laboratory evaporator.

Column Temperature Control. The vapor jacket of the column was 2 inches in diameter and about 16 inches long. It was wrapped with asbestos paper, wound with 24-gage B & S Nichrome wire, and then wrapped again with asbestos paper and aluminum foil. Electrical heating of the wire was controlled by a variable transformer. A 200-ml., round-bottomed flask, which held the reflux liquid, was heated by a Glas-Col heating mantle. The jacket temperature and consequently the column temperature (125° C.) used in the experiments reported here was obtained by refluxing the monomethyl ether of ethylene glycol (methyl Cellosolve) in the boiling flask and jacket.

Combustion Train. The combustion tube consisted of a 1/4-inch glass U-tube 6 inches long closely wound with Nichrome wire and enclosed in a metal container filled with magnesium oxide. The tube was filled with 20-mesh copper oxide and was connected to a 12-inch length of 1/4-inch diameter glass tube filled with magnesium perchlorate to remove water of combustion.

The inlet of the combustion tube was connected to the column by a 12/5 ball joint held in place by spring clamps. The heating wires surrounding the combustion tube and the column outlet were in series and were connected by porcelain wire nuts.

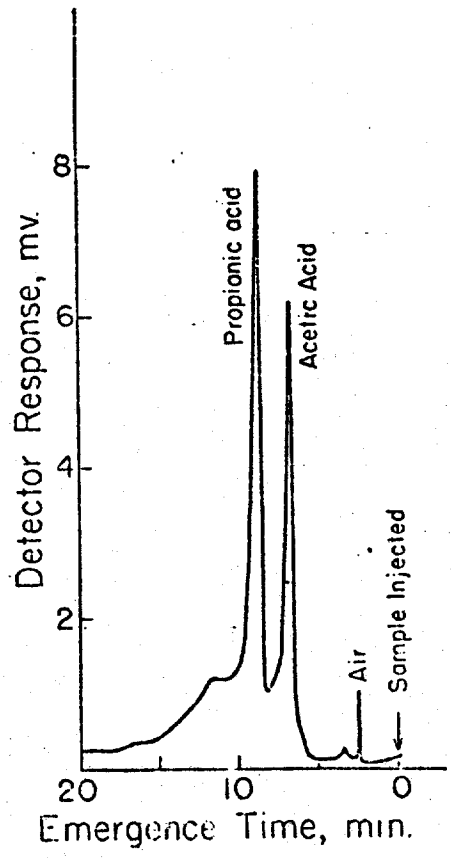

Figure 4. Chromatogram obtained by analysis of acidified solution of sodium salts of acetic and propionic acids

Sample size, 20 μl.

REAGENTS

All chemicals were reagent grade unless otherwise specified.

The acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic, and caprylic acids were commercial samples. All yielded single peaks when tested individually in the chromatographic apparatus.

The adipate polyester of diethylene glycol, which is a commercial plasticizer known as LAC-1-R 296 (Cambridge Industries Co., Inc., 101 Potter St., Cambridge 42, Mass.), was used.

Ethylene glycol monomethyl ether (methyl Cellosolve).

Helium, welding grade, 99.5% pure.

PROCEDURE

Separations were made in a column containing the adipate polyester of diethylene glycol (LAC-1-R 296) at a column temperature of 125° C., as established by the refluxing ethylene glycol monomethyl ether. (The plasticizers LAC-2-R 446, an adipate polyester of diethylene glycol that had been partially cross-linked with pentaerythritol, and LAC-3-R, composition unknown, were also found to be acceptable liquid phases.) The helium flow rate was 106 ml. per minute. The detector block was kept in air at ambient temperatures, and the filaments were operated at 200 ma. The following separations were made.

A sample containing about 0.2 mg. each of acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic, and caprylic acids was injected

onto the column to obtain the chromatogram tracing illustrated in Figure 2.

The similar tracing shown in Figure 3 was obtained by injection of an aliquot of the same mixture of acids diluted with equal volume of water. Because the higher chain acids are soluble in water to only a limited degree, it was necessary to shake the aqueous mixture vigorously during withdrawal of the sample.

Acid mixtures of unknown composition are in many cases obtained as water solutions of alkali salts extracted from biological materials. After acidification, these solutions could be sampled and treated as described above. To simulate such a situation, an aliquot of a solution of sodium acetate and propionate containing about 20% by weight of the salts was chromatographed after it was acidified to pH 2 with 85% phosphoric acid (Figure 4).

RESULTS AND DISCUSSION

Figures 2 and 3, illustrating the separation of anhydrous and hydrated organic acids, respectively, are closely similar in that the peaks occur at very nearly corresponding points in both chromatograms. Admixture of water with the organic acids used does not significantly affect the resolving power of the column under the conditions described nor does the resolving power of the column noticeably change with continued use. At the 125° C. temperature of separation selected for these runs, the more volatile components emerge as sharp, closely spaced peaks, whereas the high-boiling components are low, broad, and widely separated. This situation could be improved by operating the column at slightly higher temperatures; however, higher temperatures will cause a decrease in the resolution of the lower-boiling components. For quantitative work two determinations at different temperatures could be made. One could be run at a temperature below 125° C. to improve the separations of the C_2 through C_5 acids, and another separate determination made at a higher temperature to improve the separation of the acid homologs above C_5.

Figure 4 illustrates that the separation procedure described is applicable to the identification of simple volatile organic acids derived from plant tissues or other biological materials.

Since the liquid phases used in this work have been applied successfully in the separation of the esters of fatty acids (*3*), higher acids than caprylic could be analyzed on the same column at the same operating temperature by converting them to esters.

ACKNOWLEDGMENT

The authors thank E. B. Kester of this laboratory for technical advice.

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Separation of Polycyclic Aromatic Hydrocarbons in Complex Mixtures

Chromatographic Determination of Trace Amounts in Petroleum Waxes

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▶ Four polycyclic aromatic hydrocarbons—benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benz[*a*]anthracene, and chrysene—added to petroleum waxes both individually and combined, have been recovered at as low a concentration as 0.01 p.p.m. Recovery ranged from 35% for benzo[*a*]pyrene to 94% for dibenz[*a,h*]anthracene. The analytical method was a combination of adsorption chromatography on magnesia-Celite and subsequent paper chromatography using different solvent systems.

MANY natural and commercial products are of interest in studies of environmental cancer because they may contain very small quantities of polycyclic aromatic hydrocarbons, some of which are carcinogenic. As some petroleum products may contain traces of polycyclic aromatic hydrocarbons (not necessarily carcinogenic), methods for the isolation and estimation of those hydrocarbons added to such largely aliphatic material were studied. As representative of the more difficult type of substance which might be encountered, two petroleum waxes were chosen; it has been difficult to determine trace components in waxy materials, because of their low solubility.

Five polycyclic aromatic hydrocarbons—dibenz[*a,h*]anthracene, benzo[*a*]pyrene, benz[*a*]anthracene, chrysene, and anthracene—were added to wax at three concentrations—1, 0.1, and 0.01 p.p.m.—both separately and as a mixture, and their separation and quantitative recovery were attempted.

Adsorption chromatography seemed the best means of separating the polynuclear hydrocarbons from the nonaromatic material. The most suitable adsorbent for such a separation was magnesium oxide (calcined magnesite) mixed with Celite to permit easier filtration (*3*). Magnesium oxide is an extremely powerful adsorbent; benzene is unable to elute tetracyclic or pentacyclic aromatic hydrocarbons from it. This is important because large volumes of benzene had to be washed through the chromatographic columns to remove the wax.

The very high adsorption affinity of magnesia for the polynuclear hydrocarbons prevented their quantitative removal from the adsorbent by simple elution with polar solvents (acetone, ethyl alcohol). Thus, from 20 g. of magnesia on which were adsorbed 100 of dibenz[*a,h*]anthracene, only 10

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powerful factor in determining whether an activity will be sought out, and whether it will be continued once initiated.

"Provided the uncertainty is not too great, so that the individual has little hope of handling it in any effective fashion, and provided that there appears to be some pattern of resolution of the uncertainty, it can be regarded as a critical characteristic of many activities"[3].

Much of the force of this theory turns on the interpretation of "effectively". The interpretation of this term is discussed in some detail in the original paper. It will be clear, however, from the outline just given, that Dr. Halliday's results would agree with the predictions from this theory, given certain additional stipulations about the nature of the "information" and the degree of sophistication of the rat. One would usually simply welcome a confirmation of a theory without further comment; however, there are two points which should perhaps be made at this stage of the research, particularly because Dr. Halliday's tasks are simpler, from an informational standpoint, than many others which might be used later, either for other organisms, or even for other rats:

(1) If the information processing theory is more adequate than the "novel stimuli" or "curiosity" form of explanation, then Dr. Halliday's results may not always be confirmed with experimental subjects of greater complication than rats, or with rats in a more complicated exploratory situation, or even with some of the rats in his study. This is important, because a repetition of the study by a different investigator with some changes in the subjects, the environment or the conditions might produce a less convincing result, thus weakening his primary conclusion —which in my opinion is sound.

(2) The slight and non-significant difference between the groups in the final 30 sec of the second trial may be more important than it appears at first sight, and Dr. Halliday may be doing his experiment less than justice in dismissing it as "too slight to merit much attention". In view of the bearing which it might have on the information processing activity it should be followed up with perhaps a larger number of subjects and/or a different method of statistical analysis.

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After Effects from Entoptic Movements

THE well known after effect of movement is normally generated by moving the images of external objects across the retina[1]. The present study shows that an after effect can also be generated by moving the retinal images of internal (entoptic) structures of the eye. These structures are normally invisible, because of selective retinal adaptation, until their shadows are artificially made to move across the retina.

Two such fixed structures are the retinal blood vessels and "Haidinger's brush".

The receptor cells lie at the back of the retina, behind the blood vessels and other structures. A receptor lying behind a blood vessel is always in shadow, so it is permanently adapted to a slightly more sensitive level than its neighbouring receptors. If the shadows are made to scan across adjacent receptors, a whole tree-like pattern of blood vessels becomes visible. This can be done by putting illumination through the sclera, and by oscillating the light source. The pattern dies away rapidly when the movement of light and shadows is stopped, presumably owing to selective adaptation.

We were the two subjects. The head was clamped by a bite bar and the left eye shaded. The real image of a bright source of light was cast on the sclera of the right eye, near the temporal margin. The image was made to scan downwards across the sclera and fly back, being suppressed during flyback, at a rate of 1 c/s. This trans-scleral light illuminated the [illegible] pattern of blood vessels was seen [illegible] upwards through an estimated 5–[illegible] The visual field was made totally [illegible] shadows of the blood vessels, by means [illegible] table tennis ball placed close to the eye, leaving a small area of sclera exposed to receive the scanning image. Unfortunately, it was impossible to exclude some parts of the stimulating apparatus from the peripheral visual field.

After scanning for 30 sec the image was held stationary on the sclera.

A pronounced downward after effect was observed, taking the form of a downward drift of the entire tree-like pattern. Both the pattern and the drifting after effect persisted for 2–10 sec.

If the image scanned the sclera upwards, the shadows were seen to move downwards and the after effect was upwards, as expected. If the image moved to the left, the shadows moved to the right and after effect was to the left, also as expected.

The after effect is not unexpected. The necessary condition is movement of images across the retina, and although the blood vessels themselves are stationary, their shadows do scan across the retina under these conditions. This result conflicts with that of Wohlgemuth[2], however, who found that uniform translatory movement of the entire visual field produced no after effect. It is just possible that in the present investigation the after effect would not have appeared without the apparatus visible in the extreme periphery, but this seems unlikely, as the after effect covered the entire visual field, not just the visual area near the apparatus.

Haidinger's brush[3] is a small shadowy object which subtends a visual angle of 2°–3°, shaped like a bow tie or spindle, which is seen on viewing a bright uniform field of plane polarized light. The plane of polarization must change continually, otherwise the brush fades in about 0·5 sec, presumably owing to selective adaptation. This phenomenon is the only known way in which the vertebrate eye can detect the polarization of light. It is probably due to unidentified dichroic structures in the fovea, perhaps in the cones themselves.

An aluminized screen was illuminated by blue polarized light. A piece of 'Polaroid' and a deep blue filter were held in the projection beam, mounted in a ball race and rotated at 1 r.p.s. On viewing the screen, the observer saw Haidinger's brush within the foveal visual field, rotating in the same direction as the 'Polaroid'. After 30 sec the rotation of the 'Polaroid' was stopped. A brief (less than 0·5 sec) but pronounced after effect of apparent rotation in the opposite direction was reported.

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AGRICULTURE

Rapid Method for Saponification of Milk Fat and Subsequent Quantitative Estimation of the Lower Fatty Acids

THE quantitative estimation of the lower fatty acids of milk fat by gas-liquid chromatography has caused some difficulty in the past. The methyl esters, particularly methyl butyrate, are undesirably volatile. The free acid-

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have low volat[illegible] and adsorption [illegible] in long retention [illegible] case, standard [illegible] In addition, the [illegible] fication of milk [illegible]

Free fatty ac[illegible] very efficient [illegible] internal diame[illegible] 20 M' terephth[illegible] of this column [illegible] been reported. [illegible] rapid method for [illegible] of the lower acid[illegible] packing.

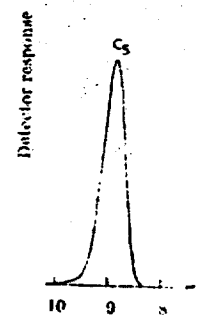

Fig. 1. Separation [illegible] acids.

An adaptation [illegible] saponify the fat [illegible] refluxed with a [illegible] potassium hydro[illegible] in a 50 ml. round [illegible] removed under [illegible] acidified, and [illegible] portions of di[illegible] concentrated at [illegible] chromatography [illegible] chromatography [illegible] in Table 1.

Excellent separ[illegible] this high efficien[illegible] measurements fo[illegible] acids were in good [illegible] 0·3 g portions of a [illegible] separately and a[illegible] chromatography. [illegible] was satisfactory in [illegible]

Table 1. CONDITIO[illegible]

Chromatograph	[illegible]
Column dimensions	[illegible]
Solid support	[illegible]
Stationary phase	[illegible]
Column temperature	[illegible]
Carrier gas	[illegible]
Detector	[illegible]
Analysis time	[illegible]

Table [illegible]

Acid	
Butyric (C 4)	[illegible]
Caproic (C 6)	[illegible]
Caprylic (C 8)	[illegible]
Capric (C 10)	[illegible]
Lauric (C 12)	[illegible]

* Standard devi[illegible]

have low volatilities which, in conjunction with association and adsorption on the column and apparatus, often result in long retention times and badly tailing peaks. In either case, standard deviations of estimates tend to be large. In addition, the traditional procedures used for saponification of milk fat are time consuming.

Free fatty acids (C_2–C_{18}) have been separated using a very efficient column (stainless steel 5 ft. × 0·055 in. internal diameter) packed with 10 per cent 'Carbowax 20 *M*' terephthalic acid on a silanized support[1]. The use of this column for quantitative investigations has not been reported. In this laboratory we have developed a rapid method for quantitative gas-liquid chromatography of the lower acids (C_4–C_{12}) of milk fat based on this column packing.

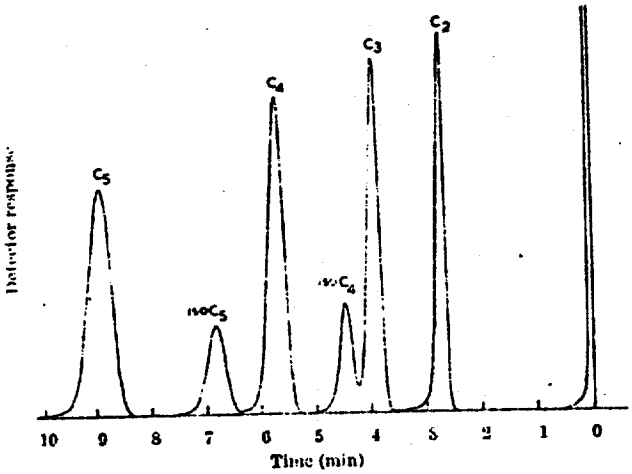

Fig. 1. Separation of short chain (C_2–C_5) acids. Temperature, 105° C; argon flow, 60 ml./min; column, see text.

An adaptation of the method of Keeney[2] was used to saponify the fat rapidly. 0·3 g of well mixed fat were refluxed with 5 ml. of a solution containing 50 mg of potassium hydroxide per ml. of isopropanol for 30 min in a 50 ml. round-bottomed flask. The solvent was then removed under reduced pressure at 80° C, the soaps acidified, and the free acids extracted with 5×1 ml. portions of diethyl ether. The ethereal solution was concentrated at 52° C and 0·7 µl. were injected on to the chromatography column. The conditions for gas-liquid chromatography of the free fatty acids (C_4–C_{12}) are shown in Table 1.

Excellent separations of C_4–C_{12} acids were obtained on this high efficiency column in 10 min, and quantitative measurements for five injections of a standard mixture of acids were in good agreement (Table 2). In addition five 0·3 g portions of a sample of butterfat were each saponified separately and analysed for C_4–C_{12} acids by gas-liquid chromatography. The agreement between measurements was satisfactory in this case also (Table 2).

Table 1. CONDITIONS FOR CHROMATOGRAPHY OF FREE ACIDS C_4–C_{12}

Chromatograph	Pye panchromatograph.
Column dimensions	5 ft. × 0·4 cm internal diameter glass.
Solid support	60/80 mesh acid-washed, dimethyl dichlorosilane-treated 'Chromosorb W'.
Stationary phase	20 per cent 'Carbowax 20 *M*' terephthalic acid.
Column temperature	235° C.
Carrier gas	Argon at 75 ml./min.
Detector	Flame ionization.
Analysis time	About 10 min.

Table 2. COMPOSITION OF FATTY ACID MIXTURES

Acid	Standard mixture Mean area per cent	S.D.*	Acids from butterfat Mean area per cent	S.D.*
Butyric (C_4)	19·81	±0·38	18·98	±0·23
Caproic (C_6)	10·03	±0·07	15·33	±0·31
Caprylic (C_8)	9·92	±0·38	10·03	±0·13
Capric (C_{10})	30·73	±0·30	31·21	±0·15
Lauric (C_{12})	29·47	±0·47	31·32	±0·21

* Standard deviation.

Although our quantitative work has been limited to milk fat we have noticed that this column is effective at lower temperatures in separating C_2–C_5 acids, including iso-C_4 and iso-C_5 (Fig. 1). With longer columns and a smaller proportion of stationary liquid to solid support it should be possible to obtain complete separation of iso-C_4 from C_3 in a very short time. Packed columns with this very stable stationary phase could then be used to give rapid quantitative separations of rumen acids.

Further details of this work will be published elsewhere.

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ANTHROPOLOGY

Africa and Pleistocene Overkill?

DR. MARTIN's paper[1] calls for comment, both on questions of fact and of interpretation, at least in regard to what he writes about Africa.

It is a pity that he bases his fossil data on the book by Cooke[2], which was written in 1963 and published before the volume on Olduvai Gorge (published in 1965)[3], in which there was revision of some earlier studies as well as the publication of various new genera and species. Dr. Martin does give this latest volume in his list of references, but his only mention of it in the text appears to be "In Africa . . . certain authors have regarded drought as the cause of Late Pleistocene extinction" followed by a very summary dismissal of the idea. Moreover, there is no reference, either in the list of references or in the text, to the work of the two leading authorities on African Pleistocene faunas—Professor Arambourg of Paris and Professor Deitrich of Berlin.

We are told that the lists of African genera shown in Table 2 consist of extinct and living African genera that are "estimated to exceed 50 kg adult body weight", but, when we turn to the table, we find in Part A *Cercopithecoides*, *Parapapio* and *Lycyaena*. What evidence is there that these two primates and this rather small hyaena weighed 50 kg? A large male *Papio* of any species is almost certainly heavier than either *Cercopithecoides* or *Parapapio* could have been, but the author does not allow *Papio* in Part C of his table. It would seem unlikely that *Lycyaena* ever attained a body weight of 50 kg any more than *Hyaena* does today, but *Hyaena* is listed in Part C. The spotted hyaena *Crocuta* certainly seems to qualify, but it is a much more heavily built animal than the striped hyaena. One wonders whether there is any good reason to believe that *Australopithecus* weighed as much as 50 kg. It is also doubtful if it should be listed as a distinct genus from *Paranthropus*. Most people now allow the latter only sub-generic rank.

Turning to Part B of the list, we find *Stylohipparion* and *Eurygnathohippus* treated as two separate genera. This ignores the evidence which was published in 1965 (ref. 3) that they represent one and the same species. Furthermore, a number of genera which are on record from East African Middle Pleistocene deposits and which became extinct at the end of Bed IV times are omitted. Presumably this is because Cooke's outdated list[2] was relied on. For example, *Nesocephalus*, *Thalerocerus* and *Parmulacius* are all missing while *Paltiphagomoides* is listed in Part A, whereas it was shown clearly, in the Olduvai volume[3], that it is a creature which persists into the period covered by Part B. In Part A genera such as *Nyanzachoerus*, *Ectopotamochoerus*, *Promesochoerus*, *Menelikia*, are all ignored, which puts the whole picture out of balance. If

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PORTAL ABSORPTION OF FATTY ACIDS IN LYMPH- AND PORTAL VEIN-CANNULATED RATS

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SUMMARY

The route and the rate of the intestinal absorption of [1-^{14}C]oleic, [1-^{14}C]-caprylic, and 2-[^{14}C]ethyl-*n*-caproic acids have been studied using lymph- and portal vein-cannulated rats. It was found that 85% of absorbed oleic acid was transported *via* lymphatic system. However, as much as 15% of absorbed oleic acid was transported directly *via* the portal system. With short-chain fatty acids, between 94–98% of the absorbed acids were transported *via* the portal system. Studies on the rate of the intestinal absorption of these acids indicated that 2-ethyl-*n*-caproic acid was absorbed less completely and subsequently metabolized less effectively than the corresponding straight-chain fatty acid, caprylic acid.

Studies on the distribution of radioactivity in lymph lipids showed that most of the radioactivity (85%) was present as triglycerides when [^{14}C]oleic acid was administered to lymph and portal vein fistula rats. However, when the ^{14}C-labeled short-chain fatty acids were given, 96–102% of the radioactivity was present as free fatty acids.

Studies on the distribution of radioactivity in lipid fractions of portal vein blood showed that 50 and 98–100% of the radioactivity present were in the form of free fatty acids when [^{14}C]oleic acid and ^{14}C-labeled short-chain fatty acids, respectively, were administered to lymph and portal vein fistula rats.

INTRODUCTION

It has been reported that long-chain fatty acids, when administered to rats, are absorbed *via* the lymphatic system mainly in the form of triglycerides in chylomicrons[1–8], while short-chain fatty acids are absorbed *via* the portal system and appear there primarily as the free fatty acids[9–11]. However, after administration of radioactive long-chain fatty acids to lymph-fistula rats, small amounts of $^{14}CO_2$ were found in expired air, suggesting possible portal absorption of these fatty acids[1,6*]. According to BORGSTRÖM[12], only about 25% of the absorbed ^{14}C activity appeared

* R. H. COOTS, personal communication.

in the intestinal lymph after the administration of [1-^{14}C]palmitic acid to bile-fistula rats. He suggested that bile salts may effect fat absorption not only by their detergent action in the intestinal lumen, but by directly affecting the metabolism of the mucosal cells, and thereby altering the route and form of fatty acid transport. Recently, SAUNDERS AND DAWSON[13] reported that about one-half of the fed ^{14}C activity was absorbed when uniformly ^{14}C-labeled oleic acid was administered to bile-fistula rats. Moreover, only about 7% of the absorbed ^{14}C activity was recovered in the lymph lipids, and the amount of unesterified fatty acid present in the lymph was 3 to 4 times that in normal animals. These results suggested that in the absence of bile, there is an altered route of transport of absorbed oleic acid as well as some failure in the esterification of the fatty acid. These authors pointed out that, although long-chain fatty acids are primarily transported *via* the lymphatic system after esterification in the mucosa, it was likely that some long-chain fatty acid is normally absorbed *via* the portal vein.

Thus, indirect evidence, such as incomplete recovery of absorbed long-chain fatty acids in lymph[4-8], the presence of $^{14}CO_2$ in expired air of lymph-drained animals fed isotopic long-chain acids[1,6*], and the appearance of radioactivity in portal vein blood of bile-fistula animals[14] have raised some questions regarding the exclusive lymphatic transport of long-chain fatty acids.

In the present experiments, the route and rate of the intestinal absorption of [1-^{14}C]oleic acid, [1-^{14}C]caprylic acid and 2-[^{14}C]ethyl-*n*-caproic acid were studied in lymph- and portal vein-fistula rats over an 8-h period. The lipid distribution in lymph and portal vein blood of the absorbed fatty acids was also determined to provide additional information with respect to the significance of portal absorption of long-chain fatty acids.

MATERIALS AND METHODS

Materials

[1-^{14}C]Oleic acid and sodium [1-^{14}C]caprylate were purchased from the Volk Radio Chemical Company and were diluted with analytically pure (99%) oleic and caprylic acids purchased from Hormel Institute, University of Minnesota. 2-[^{14}C]-ethyl-*n*-caproic acid was synthesized using [1-^{14}C]ethyl bromide and diethylbutyl malonate by the scheme on page 298.

The product contained 10–15% caproic acid and was further purified by distillation and silicic acid chromatography. The final product was obtained 98–99% pure, based on thin-layer chromatography and on refractive index, in comparison with authentic 2-ethyl-*n*-caproic acid. The radiopurity of the three acids was further checked by addition to cold samples of lymph and portal blood and subsequent analysis by the detailed procedures outlined below.

Animals

Adult male rats of Carworth Farm's Wistar strain, weighing 250 to 300 g were maintained on standard laboratory chow and water *ad libitum* prior to operation. The thoracic duct was cannulated by a modification of the procedure of BOLLMAN, CAIN AND GRINDLAY[15]. After completion of the thoracic duct cannulation, the liver and left kidney were replaced in their original positions. The spleen was then freed from the stomach by removing the mesentery between these two organs. The lienal

portal vein but did not block portal blood flow. The ligature was then tightened. Care was taken during the entire procedure not to damage the pancreatic tissue in the gastrosplenic ligament. A small amount of heparin (0.01–0.2 ml, 1000 units per ml) was placed in the tubing to prevent blood clotting. For collection of samples which were used in section 2, distilled water was substituted for heparin. The external end of the tubing was then blocked with a needle. The needle was removed from the tubing only when blood samples were taken. Finally, all blood vessels connected to the spleen were tied off individually to avoid any possible damage to the pancreas, and the spleen was removed. The viscera were then replaced. During the entire period of the operation, the organs were kept moist with warm physiological saline. The final position of the cannulae and tissues are shown in Fig. 1. The animal was placed in a restraining cage designed for collection of feces as well as lymph and blood samples. All animals were allowed free access to saline, but no food for 48 to 50 h prior to the administration of the test emulsions. The test emulsions were prepared immediately before use, as described by VAHOUNY, FAWAL AND TREADWELL[16]. Each of the rats in all groups was given 3 ml of the appropriate test emulsion intragastrically using light vinyl ether anesthesia. After administration of the test emulsion, lymph was collected in 2-h periods for 8 h in tubes containing 0.05 ml of heparin, and 0.1 ml of portal vein blood was collected at 0.25, 0.5, 1, 2, 4, 6 and 8 h. At the end of the experiment, the animals were sacrificed, the stomach and intestine were removed, flushed with saline, and the washings combined with the fecal samples. The combined feces and intestinal contents, and the small intestine were homogenized in 15 ml isotonic saline.

Methods

Aliquots of lymph and blood samples and of the homogenates were extracted with 24 volumes of ethanol–ether (3:1, v/v). 1–2 ml of the ethanol–ether extract was then transferred into a counting vial, 10 ml of liquid scintillant (100 mg 1,4-di-2(5-phenyl oxazolyl)-benzene + 4 g 2,5-diphenyl oxazole per 1000 ml toluene) was added and the radioactivity measured in a liquid scintillation counter (Nuclear Chicago Series 720, 3-channel counter).

For the determination of the form of radioactivity present in the lymph lipids, the lymph samples (4 time samples were taken from each animal) were pooled and total lymph lipids were extracted with 24 volumes of ethanol–ether (3:1, v/v). An aliquot of the ethanol–ether extract was then placed to a large separatory funnel, equal volumes of solution A (distilled water saturated with petroleum ether and diethyl ether and containing 1% $NaHCO_3$ and 0.58% NaCl) and solution B (petroleum ether saturated with distilled water) were added, the contents mixed and allowed to separate. The petroleum ether upper layer was separated from the lower layer and the aqueous layer was returned to the separatory funnel. The same amount of solution B was added, mixed and the upper layer was again separated from the lower layer. The petroleum ether layers contained all of the lymph lipids except free fatty acids and the lower aq. layer contained sodium salts of fatty acids. The petroleum ether layers were then pooled, evaporated to dryness, and the residue dissolved in 30–40 ml of warm petroleum ether. The petroleum ether solution was dried over Na_2SO_4 overnight and the lymph lipids were fractionated by silicic acid column chromatography using a modification of the procedure of HIRSCH AND AHRENS[17].

Aliquots of the total lipid, petroleum ether, and aqueous extracts, and each lipid fraction were taken for the determination of radioactivity.

For the determination of the form of radioactivity present in portal vein blood after the administration of test emulsions containing ^{14}C-labeled fatty acids the samples were treated as follows: 1 h after the administration of the test emulsion, 2 to 3 ml of blood was taken from portal vein using an oxalated syringe. The blood samples were then processed as described above except the lipids were fractionated by thin-layer silicic acid chromatography[18] using a solvent system of hexane–acetone–acetic acid (90:11:3, v/v/v). In all experiments, apparent differences were analyzed for significance using the *t*-test[19].

RESULTS AND DISCUSSION

Before studying the portal absorption of fatty acids, it was necessary to demonstrate the absence of lympho-portal and lympho-vena cava anastomoses in the experimental animals. Two lymph- and portal vein-fistula rats were each given 15 μC [1-^{14}C]oleic acid (292 mg) and 50 μC [7-^{3}H]cholesterol (50 mg), which is known to be absorbed exclusively *via* the lymphatic system. 3 h after the administration of the test emulsion, 2–3 ml of portal vein blood was removed and the radioactivity present was determined. If 0.001% of the administered cholesterol had been transported to the circulatory system by any of the above channels, it could have been easily detected in the portal blood samples. Actually the data showed that whereas a considerable amount of ^{14}C activity was present in the portal vein blood, no ^{3}H activity above background could be detected. Also, in order to obtain additional evidence for portal absorption under normal conditions, a test emulsion containing [1-^{14}C]oleic acid was administered to two lymph and portal vein fistula rats. 1 h later simultaneous samples of portal and inferior vena cava blood were taken and the ratio of lipid radioactivity in the portal blood to inferior vena cava blood was determined. The ratios obtained were 1.5 and 3.2 in the two animals, again indicating absorption and transport *via* the portal system. From these results, it was concluded that ^{14}C activity recovered from portal vein blood after the administration of ^{14}C-labeled fatty acids to the experimental animals was due to direct absorption *via* the portal system.

The rate and route of the intestinal absorption of fatty acids

The rate and route of the intestinal absorption of fatty acids were studied by administering ^{14}C-labeled fatty acids and following the serial appearance of radioactivity in thoracic duct lymph and the portal vein blood. The compositions of the test emulsions used are shown in Table I. The three groups of rats shown in Tables II–III and Figs. 1, 2 are numbered corresponding to the emulsions administered.

As shown in Table II, when [1-^{14}C]oleic acid was administered to lymph and portal vein fistula rats, about 62% of the fed radioactivity was recovered in thoracic duct lymph during the 8-h experimental period, while 23% was unabsorbed and was recovered in feces and intestinal contents. In the groups fed ^{14}C-labeled short-chain acids (Groups 2 and 3), less than 4% of the fed radioactivity was recovered in thoracic duct lymph in 8 h. With [1-^{14}C]caprylic acid 5% of the isotope was recovered in the combined feces and intestinal contents, and less than 1% remained in the intestinal wall at 8 h. However, in the group fed 2-[^{14}C]ethyl-*n*-caproic acid, 48% of the fed

TABLE I

COMPOSITION OF THE INTRAGASTRIC TEST EMULSION

Emulsion number	*1*	*2*	*3*
Albumin, 50 mg	+	+	+
Sodium taurocholate, 288 mg	+	+	+
[1-^{14}C]Oleic acid, 292 mg (4.0 μC)	+		
[1-^{14}C]Caprylic acid, 150 mg (3.0 μC)		+	
2-[^{14}C]Ethyl-*n*-caproic acid, 150 mg* (3.2 μC)			+
0.9% Saline to 3 ml	+	+	+

* ^{14}C label was on the side-chain as shown: $CH_3CH_2CH_2CH_2CH(^{14}CH_2CH_3)COOH$

TABLE II

RECOVERY OF RADIOACTIVITY AFTER THE ADMINISTRATION OF ^{14}C-LABELED FATTY ACIDS

Apparent portal absorption was calculated by subtracting the percentage of ^{14}C activity present in thoracic duct lymph, small intestine, and feces and intestinal contents from 100.0%.

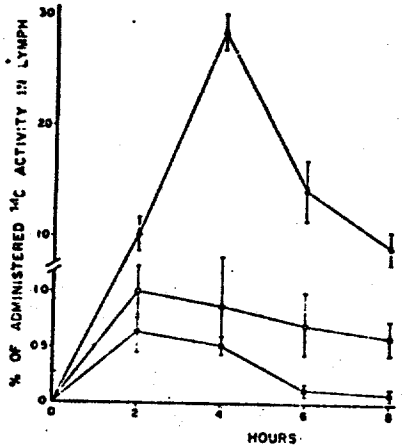
Lymph (8 h) (%)	*Small intestine (%)*	*Feces and intestinal contents (%)*	*Apparent portal absorption (%)*
*Group 1: 292 mg [^{14}C]oleic acid**			
61.9 ± 6.9	3.6 ± 0.8	23.1 ± 2.0	11.3 ± 0.4
Group 2: 150 mg [^{14}C]caprylic acid			
1.4 ± 0.2	0.6 ± 0.1	5.0 ± 0.3	93.0 ± 0.2
Group 3: 150 mg 2-[^{14}C]ethyl-n-caproic acid			
3.2 ± 1.0	1.2 ± 0.4	47.6 ± 5.1	49.8 ± 5.1

* All emulsions contained 50 mg albumin + 288 mg sodium taurocholate to 3 ml with saline, in addition to the designated fatty acids.

isotope was unabsorbed in 8 h, while about 1% was present in the intestinal wall. These data clearly indicate that the straight-chain acid is more rapidly absorbed in the rat than the 8-carbon branched-chain analogue.

The difference between the radioactivity administered and that recovered (sum of per cent recovered from lymph, small intestine, and combined feces and intestinal contents) gives an approximation of absorption of the fatty acids by a route other than thoracic duct lymph, presumably via portal blood. These data (Table II, apparent portal absorption) show that about 93 and 50%, respectively, of the administered radioactivity appeared in the portal vein blood after the administration of caprylic and 2-ethylcaproic acids. It is of particular interest that these data show that about 11% appeared in portal vein blood when [1-^{14}C]oleic acid was given. The results clearly indicate that a major portion (84.5%) of the labeled oleic acid which left the gut wall was absorbed *via* the lymphatic system. The remainder was transported *via* the portal vein, and as discussed above, this amount was not due to lympho-venous anastomoses.

The rates of appearance of absorbed fatty acids in thoracic duct lymph are shown in Fig. 2. Administration of the aqueous emulsions of ^{14}C-labeled fatty acids to these animals results in a peak of absorption between 2 and 4 h after feeding and a subsequent decline in the appearance of isotope.



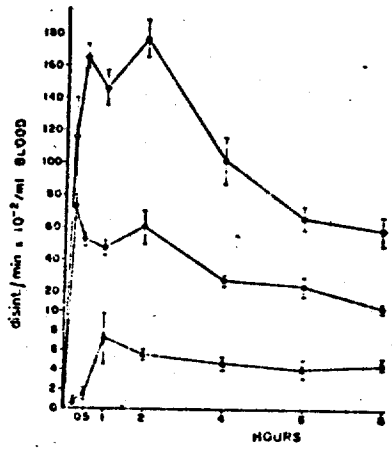


Fig. 2. Rate of appearance of radioactivity in thoracic duct lymph following administration of test emulsions. Emulsion 2: 50 mg albumin + 288 mg sodium taurocholate (control) in addition to 292 mg [^{14}C]oleic acid (4.6 μC); Emulsion 3: Control + 150 mg [^{14}C]caprylic acid (3.0 μC); Emulsion 4: Control + 150 mg 2-[^{14}C]ethyl-*n*-caproic acid (3.2 μC). Values are expressed as per cent of radioactivity recovered in the lymph and represent means of four animals ± S.E. Lymph samples were collected in 2-h periods for 8 h. ▲——▲, [1-^{14}C]oleic acid; ○——○, 2-[^{14}C]ethyl-*n*-caproic acid; ●——●, [1-^{14}C]caprylic acid.

Fig. 3. The appearance of radioactivity in portal blood system following administration of test emulsion. See Table I for composition of test emulsions. Values are expressed as desintegrations/min present per ml of portal vein blood at a given time and represent means of four animals ± S.E. ○——○, 2-[^{14}C]ethyl-*n*-caproic acid; ●——●, [1-^{14}C]caprylic acid; ▲——▲, [1-^{14}C]oleic acid.

Fig. 3 summarizes analysis of portal vein blood after administration of the labeled fatty acids. Since these analyses were carried out on blood from animals with complete thoracic duct lymph drainage, appearance of isotope gives a direct indication of portal transport of fatty acids. With the short-chain acids, caprylic and 2-ethyl-*n*-caproic acid, large amounts of the isotope were present in portal blood within 15 min and the levels reached a peak 15 and 30 min after administration, indicating the very rapid absorption of these acids. The second peak at 2 h may not represent true peaks for portal absorption of these acids. Since by 2 h the blood had circulated several times, radioactivity detected at this time is a combination of the radioactivity already present in the circulation and the radioactivity still being transported from the intestine.

It is also interesting to note that when a similar total dose of radioactivity was given either as [^{14}C]caprylic acid (3.0 μC) or as 2-[^{14}C]ethyl-*n*-caproic acid (3.2 μC), there was two to three times as much ^{14}C-activity present in portal vein blood after 2-ethyl-*n*-caproic acid as after caprylic acid. These high levels of radioactivity in the portal system could be the result of either rapid transport of the branched-chain acid from the intestine to portal vein blood or to slow removal of the absorbed acid from the circulation by other tissues, *i.e.* the liver. Since only about 50% of the fed 2-[^{14}C]-ethyl-*n*-caproic acid was absorbed from the intestine while more than 90% of the fed [^{14}C]caprylic acid was absorbed, the higher levels of radioactivity found in portal blood after the administration of 2-[^{14}C]ethyl-*n*-caproic acid are likely due to the slower clearance of this acid from the general circulation.

The data presented above clearly indicate that short-chain fatty acids, such as

caprylic and 2-ethyl-*n*-caproic acids, are absorbed almost exclusively *via* the portal system (94–98%), and only a small amount (less than 3%) of the fed acid appears in thoracic duct lymph. When [^{14}C]oleic acid was given to lymph and portal vein fistula rats, a major portion of the absorbed acid was recovered in thoracic duct lymph (85%). However, up to 15% of the absorbed oleic acid was transported *via* the portal system. Recently, SAUNDERS AND DAWSON[20] reported that a large proportion of oleic acid is transported *via* the portal system when bile is diverted from the intestine. They suggest that this alteration in the route of transport of absorbed oleic acid in bile-fistula rats may represent a fuller utilization of a minor pathway in normal rats. The data presented in this paper suggest that the portal route plays a significant role in the transport of absorbed long-fatty acids in normal animals. This route of long-chain fatty acid transport may be even more significant when there is a failure in the esterification of these acids in the intestinal mucosa.

Distribution of radioactivity in lipid fractions of thoracic duct lymph and portal vein blood after administration of labeled fatty acids

Distribution of radioactivity in lipid fractions of thoracic duct lymph. After pooling the time samples of thoracic duct lymph, the lymph lipids were extracted and fractionated by silicic acid column chromatography. The per cent distribution of radioactivity in the different fractions is summarized in Table III.

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN LIPID FRACTIONS OF THORACIC DUCT LYMPH

Cholesterol ester (%)	*Triglyceride (%)*	*Minor lipids (%)*	*Free fatty acids (%)*	*Phospholipid (%)*
*Group 1: 292 mg [^{14}C]oleic acid**				
1.1 ± 0.1	85.4 ± 3.1	3.9 ± 1.1	7.8 ± 2.2	1.8 ± 0.3
Group 2: 150 mg [^{14}C]caprylic acid				
0.0	28.7 ± 4.0	2.3 ± 0.7	69.5 ± 4.3	0.0
Group 3: 150 mg 2-[^{14}C]ethyl-n-caproic acid				
0.0	7.9 ± 1.2	1.0 ± 0.3	90.4 ± 1.4	0.0

* All emulsions contained 50 mg albumin – 288 mg sodium taurocholate to 3 ml with saline, in addition to the designated fatty acids. The values represent means ± S.E. of lipid fractions in 8-h samples of lymph.

In Group 1, which received [1-^{14}C]oleic acid, 85% of the radioactivity was present in the form of triglycerides and 2 to 4% appeared as cholesterol esters and phospholipids. About 8% was present as free fatty acid. This data is comparable to that reported by BORGSTRÖM AND TRYDING[11]. In contrast to [^{14}C]oleic acid, when [^{14}C]caprylic acid or 2-[^{14}C]ethyl-*n*-caproic acid were administered, most of the radioactivity was present as free fatty acid (69.5 and 90.4%, respectively). No radioactivity was present in the cholesterol ester fraction, indicating that neither caprylic acid or 2-ethyl-*n*-caproic acid was directly involved in the formation of lymph cholesterol esters. Furthermore, only 8 to 29% of the radioactivity was in the form of triglyceride. These results with short-chain acids differ from those of BLOMSTRAND[8]

who found that most of the radioactivity recovered in the lymph was present as triglycerides after the administration of [^{14}C]decanoic acid.

In the case of the short-chain fatty acids, the appearance of a small amount of ^{14}C activity in thoracic duct lymph may or may not represent true absorption *via* the lymphatic system. It is possible that the small amount of ^{14}C activity was transferred from the blood to lymph during the course of general circulation. However, it has been reported by BLOMSTRAND[10] that free decanoic acid is not able to pass the blood-lymph barrier and enter the thoracic duct lymph.

Distribution of radioactivity in lipid fractions of portal vein blood. Having established that 12 to 15% of the radioactivity of absorbed [^{14}C]oleic acid is transported *via* the portal system, it was decided to study the distribution of radioactivity in the lipid fractions of portal vein blood after the administration of ^{14}C-labeled fatty acids. Lymph and portal vein fistula rats were given ^{14}C-labeled fatty acids with albumin and sodium taurocholate as in Groups 1–3. (Total doses of ^{14}C activity were 15, 3.0, and 3.2 μC of oleic, caprylic and 2-ethyl-*n*-caproic acids, respectively.) 1 h after the administration of the test emulsions, 2–3 ml of portal vein blood were collected. The blood lipids were then extracted and the lipid fractions were separated by thin-layer silicic acid chromatography[17]. As shown in Table IV, when [^{14}C]oleic acid was administered, 50% of the radioactivity present in portal vein blood was in the form of free fatty acid. However, there were significant amounts of radioactivity present in the monoglyceride, diglyceride, triglyceride and phospholipid fractions. In contrast to [^{14}C]oleic acid, when [^{14}C]caprylic acid was given, essentially all of the radioactivity (96.5–99.9%) was in the form of free fatty acid; only about 2% was present as esterified fatty acid. When 2-[^{14}C]ethyl-*n*-caproic acid was administered, all of the radioactivity present in portal vein blood was in the form of free fatty acid (98.9–102.2%). These results are contrasted to those reported by BORGSTRÖM[11] with [^{14}C]decanoic acid in which 64–74% of the fed ^{14}C activity was present in the form of free fatty acid in portal vein blood.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY PRESENT IN PORTAL BLOOD AFTER ADMINISTRATION OF TEST EMULSIONS CONTAINING ^{14}C-LABELED FATTY ACIDS

The radioactivity was assayed as described in the text. Values are expressed as per cent of total radioactivity recovered in portal vein blood after the administration of ^{14}C-labeled fatty acids. Each fatty acid was administered to two animals.

Lipid fractions	*Received [^{14}C]oleic acid (%)*		*Received [^{14}C]caprylic acid (%)*		*Received 2-[^{14}C]ethyl-n-caproic acid (%)*	
	Rat 1	*Rat 2*	*Rat 3*	*Rat 4*	*Rat 5*	*Rat 6*
Phospholipids	19.1	14.9	0.6	0.3	0.0	0.0
Monoglycerides	9.9	14.4	0.0	0.6	0.0	0.0
Diglycerides	10.7	10.5	0.4	0.4	0.0	0.0
Free fatty acid	49.0	49.6	96.5	99.9	98.9	102.2
Triglycerides	9.9	7.2	1.0	0.7	0.0	0.0
Cholesterol ester	1.3	3.5	0.2	0.3	0.0	0.0

The results obtained in this experiment suggest that long-chain fatty acids and short-chain fatty acids follow different metabolic pathways after they are transported *via* the portal system. 1 h after the administration of [^{14}C]oleic acid, as much as 50% of the radioactivity recovered in portal blood was present as esterified fatty acid.

It is possible that a large portion of the oleic acid which is transported *via* the portal system is taken up by the liver and rapidly esterified. In case of short-chain fatty acids, most of the acid present in the portal system was in the form of free fatty acid, indicating that these acids are not esterified effectively by enzymes present in the liver and other tissues. It is likely that these acids, when transported *via* the portal system, are removed from the circulation, most probably by the liver, and catabolized to carbon dioxide, or are excreted in the urine.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. R. H. COOTS, Procter and Gamble Company, for access to a manuscript in the press, and to Dr. F. M. MATTSON, Procter and Gamble Company, for authentic samples of mono- and diglycerides as standards for thin-layer chromatography. This work was supported by grants from the National Heart Institute (HE-02033) and the Life Insurance Medical Research Fund.

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TECHNICAL NOTES

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Major Free Fatty Acids in Gouda Cheese

Free fatty acids (FFA) more than likely contribute to the flavor and odor of Gouda cheese. A complete analysis of the FFA in Gouda cheese is not available in the literature. Simonart and Mayandon (6) studied the aliphatic acids in Gouda cheese and reported no acid higher than acetic. Ystgaard et al. (8) determined the volatile acetic, propionic, and butyric acids in Norwegian Gouda, using steam distillation followed by column chromatography. Nakanishi et al. (5) used gas-liquid chromatography to study the FFA in Japanese Edam, Gouda, and Cheddar cheeses. The total FFA in their cheese varied from 855 to 1,563 mg per kilogram cheese. Most of the samples contained acetic, n-butyric, caproic, caprylic, and capric acids. Some Gouda and Cheddar samples contained relatively large amounts of n-valeric and iso-valeric acids. This investigation was undertaken to determine the major FFA in some American Gouda samples.

The four Gouda samples in this study were small retail packages purchased from different local markets. Each sample was ground and blended thoroughly prior to analysis.

The column of Wiseman and Irvin (7), as adapted by Bills and Day (1) for Cheddar analysis, was used for the formic acid [illegible]tic acid analyses. The acetic acid eluate was a very faint yellow, indicating some annatto coloring matter or natural carotenoid content. Assuming that the color is norbixin, the major coloring component of cheese color, and that the annatto was added at a level of 1 oz per 100 lb of cheese, any correction for bixin or norbixin is negligible, since commercial annatto color contains the equivalent of only 1.4% norbixin (2, 4). At most, this would mean an error of about 0.8 to 1.6% for the lowest value of acetic acid.

Analyses for butyric through linolenic acids were by the method of Iyer et al. (3), as used for Provolone cheese.

Table 1 indicates some variation in the FFA content of the Gouda cheese. Variation and amounts of individual acids are very similar to analyses of Bills and Day (1) for Cheddar cheese. In contrast, small amounts of formic acid appeared to be evident in the Gouda cheese. This was characterized by a very faint band in the chromatographic column. Similarity between Gouda and Cheddar cheeses is not surprising, in view of the similar starter microorganisms, ripening conditions, salt content, pH at two days, and fat.

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TABLE 1
Average of duplicate analysis of free fatty acids in Gouda cheese*

Acid	Cheese sample A	B	C	D
1:0	22	32	35	68
2:0	408	986	1,073	186
4:0	69	91	101	57
6:0	22	62	51	42
8:0	15	36	31	28
10:0	38	57	44	49
12:0	84	109	84	84
14:0	155	252	154	209
16:0	467	591	432	527
18:0	193	277	250	260
18:1	370	751	552	541
18:2	64	141	98	91
18:3	42	69	34	48
Total	1,949	3,454	2,939	2,190

* Mg acid/kg cheese.

Acknowledgment

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Dissert. Abst. 20(12): 4477 (1960)

One replication of Holstein cows was attained over the treatments each of 3 winter feeding periods with the exception of one treatment the second year, whereas the Brown Swiss breed was not replicated and was unevenly distributed throughout the years. Statistical interpretation was based upon the Holstein replications.

Actual milk, fat-corrected milk and production of milk constituents were lower for cows fed at the low level than for cows at the other feeding levels. Less production was also obtained from cows consuming the high hay (75:25) ration than from cows receiving the ratios containing more grain. This was attributed largely to the inability of the cows on the high-hay ration to consume their allotted rations. Body weight changes after the first 2 weeks of the experimental period were independent of treatment effects.

Protein percentage was higher in the milk of animals on high grain rations. However, both protein and lactose percentages in the milk were affected to a greater extent by feeding level than by ratio of hay to concentrate; as plane of nutrition increased, the levels of these constituents increased. Neither ratios nor feeding levels significantly affected fat percentage. Differences in the means for fat percentage were rather substantial, but not significant because variation within groups of cows was great.

Production efficiencies based upon a net efficiency calculation representing the energy produced in the milk per therm of feed energy less the maintenance allowance were 0.946, 1.050 and 1.154 for high, medium and low feeding levels, respectively. No appreciable differences were noted in efficiency of utilization of the various hay to concentrate ratios. A similar efficiency calculation based upon digestible energy showed increased efficiency with decreased feeding intensity (0.412, 0.457 and 0.508, respectively) and increased proportion of grain in the ration (0.403, 0.421 and 0.522, respectively). The efficiency calculation was found to be dependent upon the productive ability of the cow although it was independent of body weight changes in this study.

Microfilm $2.50; Xerox $7.00. 149 pages.

THE EFFECTS OF ISOVALERIC, CAPROIC, HEPTYLIC, AND CAPRYLIC ACIDS AS DIETARY FAT SOURCES ON THE PROTEIN AND ENERGY METABOLISM OF THE RAT.

(L. C. Card No. Mic 60-1623)

Richard Patrick King, Ph.D.
Iowa State University of Science and Technology, 1960

Supervisor: Wise Burroughs

Three body balance studies of 56-, 42-, and 35-days duration, respectively, were conducted with a total of 72 rapidly growing male albino rats to determine the possible effects of isovaleric, caproic, heptylic, and caprylic acids upon the protein and energy utilization by the rat.

The addition of 8 per cent of the sodium salts of isovaleric, heptylic, and caprylic acids to diets adequate in essential fatty acids altered the normal nitrogen and energy utilization patterns of rats, but did not affect their ability to efficiently utilize the protein and energy of the diet.

Sodium isovalerate was utilized by the rat to the same extent as was ethyl caproate in diets adequate in essential fatty acids.

The addition of 8 per cent sodium isovalerate to an otherwise fat-free diet increased the rate of development of an essential fatty acid deficiency in rats from 10-12 weeks to 2-3 weeks. The fat-deficiency was associated with a decreased nitrogen gain, but not body fat gain, and an increased heat production.

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OPTIMUM LENGTH OF THE FEEDING PERIOD FOR SELECTION OF BEEF CATTLE

(L. C. Card No. Mic 60-1836)

Louis Andre Swiger, Ph.D.
Iowa State University of Science and Technology, 1960

Supervisor: L. N. Hazel

The length of the feeding period necessary for selecting beef cattle accurately for their genetic ability to gain weight was studied. Birth weight, weaning weight and gain in each of five successive 28-day periods of a post-weaning feeding test were studied. The data came from 832 Hereford calves by 23 sires in an Ohio herd.

The environmental variables year, sex, age of dam and age of calf at weaning were examined for effects and interactions. The interaction between age of calf and sex was important. The interaction between age-of-dam and year was surprisingly large. This could have been due to confounding with sires, confounding of first and second calf three-year-old cows with years, or a true interaction between these variables. Least squares constants for the important environmental effects were computed and were used to correct the data.

Phenotypic, genic and environmental variances and covariances were estimated from paternal half-sib analyses of variance and covariance of the corrected data. These were used to compute heritability for each trait and the phenotypic, genic and environmental correlations among the seven traits. The heritabilities were generally lower than those reported in the literature. The genic correlations were quite high, the phenotypic correlations were intermediate and the environmental correlations were low or negative.

Several selection indices were computed, using various combinations of the traits. Indices were computed to predict the sum of the average effects of the genes which influence final weight at about one year of age, 140-day feedlot gain, and weaning weight. Though birth weight was a good indicator of the genic effects for these characteristics, it was of little additional value if later weights were taken. Gain in the last two feeding periods added little information about the genic effects for either final weight or post-weaning gain. Since the cost of feeding the calves an additional 56 days would be considerable, the feeding period could be shortened to 84 days with almost no loss of genetic information.

The most desirable index in selecting for final weight, considering the gain expected from selection and the cost involved, was I = .28(weaning weight) + (84-day gain).

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EFFECT OF FATTY ACIDS ON THE PROTEOLYSIS OF PROTEINS

I. EFFECT OF C_6-C_{14} FATTY ACIDS ON THE TRYPTIC DIGESTION OF OVALBUMIN

By MICHIYO KONDO

(From the Laboratory of Enzymology, Institute of Applied Microbiology, University of Tokyo, Tokyo)

(Received for publication, July 8, 1957)

It has been repeatedly demonstrated (*1*) that ovalbumin is easily digestible by enzymes after denaturation by heating, while, it is hardly digestible in the native state. The present author also reported (*2*) some results of her studies on the effects of heating on the tryptic digestibility of ovalbumin. The maximum digestion of egg white and egg albumin by trypsin was obtained by a heat treatment at 80° for 10 minutes (*2, 3*). When food protein is digested in the alimentary canal, it is usually accompanied with fatty acid. To clarify how the digestion of ovalbumin is affected by fatty acid, is interesting with respect to the physiology of digestion or enzymatic degradation of protein. The present study was designed to elucidate the effect of various fatty acids on the digestion of ovalbumin by trypsin.

EXPERIMENTALS AND RESULTS

I. Digestion Test

(*A*) *Materials:*

Crystalline Ovalbumin—Prepared by the modified method of Kekwick and Cannan. N-content 14.82 per cent.

Enzyme—Crystalline trypsin kindly supplied from Mochida Seiyaku Co. 1270 unit/mg.

Fatty Acids—Fatty acids used were *n*-caproic, *n*-caprylic, *n*-capric, *n*-lauric and *n*-myristic acids. All the acids were approximately 99 per cent pure.

(*B*) *Experiments:*

Preparation of Test Solution—An amount of 110 mg. of ovalbumin was taken into a 10 ml. conical flask and was dissolved with 2 ml. of water and 3 ml. of 0.3 *M* phosphate buffer (pH 7.8), followed by the addition of a definite amount of Na-salt of fatty acid. The final volume of the solution was made up to 10 ml. with water.

Enzyme Solution—The solution contained 5.5 mg. of crystalline trypsin in 1 ml. of water.

Denatured Ovalbumin Solution—The ovalbumin solutions were heated in a water bath (at 80° and 98°) for 10 minutes.

Tryptic Digestion—Digestion temperature: 37°, Digestion time: 1, 5, 10, 20 and 40 minutes, respectively. Three sets of 5 ml. of ovalbumin solution containing fatty acid were

Cap. acid

treated as follows:

a. control
b. heated at 80° for 10 minutes.
c. heated at 98° for 10 minuees.

Each of them was taken into 20 ml. test tube and kept at 37°. Thereupon, 1 ml. of enzyme solution was added to the test tube and mixed. At different time intervals, namely, after 1, 5, 10, 20 and 40 minutes' incubation, 1 ml. of the reaction mixture was taken out and poured into 10 ml. of 5 per cent trichloracetic solution and the resulted presipitate was filtered off.

The optical density of the filtrate was estimated with a spectrophotometer at 280 mμ, and the digestion coefficient, (TCA soluble "tyrosin"/total "tyrosin" of ovalbumin ×100) was calculated. As for a blanc test, 0.83 ml. of the substrate solution, 0.17 ml. of the engyme solution and 10 ml. of the trichloracetic acid solution were mixed at the same time and filtered. The absorbance of this filtrate was subtracted from the above estimated absorbances.

(C) *Results:*

As shown in Table I. the proteolytic activity of trypsin towards denatured ovalbumin stopped almost completely after 40 minutes incubation under these conditions. The maximum degree of digestion by longer incubation time was approximately 80 per cent. As shown in Table I, C_8 fatty acid does not affect the digestion of ovalbumin by trypsin even at the concentration of 0.4 *M*: C_8 acid, however, accelerates the digestibility of ovalbumin at 0.1 *M* to almost the same degree as the heat denatured ovalbumin, and inhibits the digestion at higher concentrations. The longer the carbon chain of fatty acid, the stronger this effect appears, and C_{12} acid has the effect for accelerating the digestion even at 0.015 *M*, and shows inhibition at 0.05 *M*. The C_{14} acid also shows the same effects, at concentrations ranging from 0.0025 to 0.015 *M*.

II. *Determination of Specific Rotation of Ovalbumin Solution*

In order to recognize whether or not ovalbumin is denatured by the addition of fatty acid, the specific rotation of ovalbumin was determined,

TABLE I

Effect of Fatty Acid Added on the Digestion of Ovalbumin by Trypsin

(a) Control (without fatty acid)

Treatment / Digestion time, (*min.*)	Digestion coefficient (%)		
	Unheated	Heated at 80°	Heated at 98°
5	2.4	26.0	24.1
10	3.5	42.0	28.5
20	4.1	50.3	34.7
40	7.5	59.2	36.7

(b) Fatty acid added

C_6 *n*-caproic acid

Concentration (*M*)	Digestion coefficient (%)											
	0.1			0.2			0.3			0.4		
Treatment / Digestion time, (*min.*)	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	3.2	34.8	29.5	2.5	—	—	2.7	—	—	2.3	—	—
10	5.8	34.3	31.0	6.1	—	—	6.5	—	—	7.5	—	—
20	7.2	43.3	37.5	7.4	—	—	8.2	—	—	7.5	—	—
40	8.1	50.3	41.3	9.2	—	—	7.6	—	—	7.5	—	—

C_8 *n*-caprylic acid

Concentration (*M*)	Digestion coefficient (%)											
	0.05			0.1			0.15			0.8		
Treatment / Digestion time, (*min.*)	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 90°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	2.5	25.2	24.6	26.8	21.9	25.5	26.2	20.3	21.0	2.3	3.5	1.2
10	3.5	38.1	30.0	30.9	33.2	30.5	35.8	31.0	28.3	1.7	2.9	3.8
20	10.8	47.5	38.1	36.0	41.3	40.0	45.1	40.2	34.8	2.0	1.9	3.6
40	8.7	57.0	46.3	40.1	51.0	46.5	50.2	49.9	40.5	0.9	6.1	5.7

C_{10} *n*-capric acid

Concentration (*M*) / Treatment / Digestion time, (*min.*)	Digestion coefficient (%)											
	0.005			0.015			0.05			0.1		
	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	5.2	32.4	24.3	20.2	32.3	28.7	29.4	28.9	28.1	12.4	19.0	10.8
10	6.1	41.4	30.1	22.2	43.4	34.4	39.6	38.6	35.4	15.4	19.5	12.6
20	6.6	58.6	37.8	27.5	55.9	42.1	47.0	42.6	37.6	17.8	21.2	17.0
40	9.1	58.4	44.9	29.9	59.9	47.5	55.0	47.2	42.7	19.2	21.1	20.2

C_{12} *n*-lauric acid

Concentration (*M*) / Treatment / Digestion time, (*min.*)	Digestion coefficient (%)											
	0.0025			0.005			0.015			0.05		
	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	9.4	34.6	27.9	17.5	40.4	29.5	34.3	41.5	37.8	9.4	7.8	10.0
10	13.0	47.0	37.9	24.2	48.4	41.4	40.8	47.5	45.3	14.5	14.5	13.4
20	16.0	56.3	45.7	35.7	57.0	46.6	44.9	50.7	47.5	17.1	16.7	20.3
40	18.9	63.0	48.4	51.0	63.8	52.7	53.5	57.5	53.0	21.8	21.1	23.8

C_{14} *n*-myristic acid

Concentration (*M*)	Digestion coefficient (%)											
	0.005			0.015			0.05			0.1		
	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°

C_{14} *n*-myristic acid

Digestion time, (*min.*)	Concentration (*M*)	0.0025		0.005		0.015		0.05	
	Treatment	Digestion coefficient (%)							
		Unheated	Heated at 80°	Unheated	Heated at 80°	Unheated	Heated at 80°	Unheated	Heated at 80°
5		11.6	39.4	15.5	39.7	32.9	50.7	13.0	13.4
10		16.7	49.6	19.9	54.0	41.2	62.5	19.5	19.9
20		26.8	59.9	22.8	61.8	44.2	70.4	24.8	24.2
40		18.9	66.9	23.3	64.9	47.4	76.9	25.1	26.3

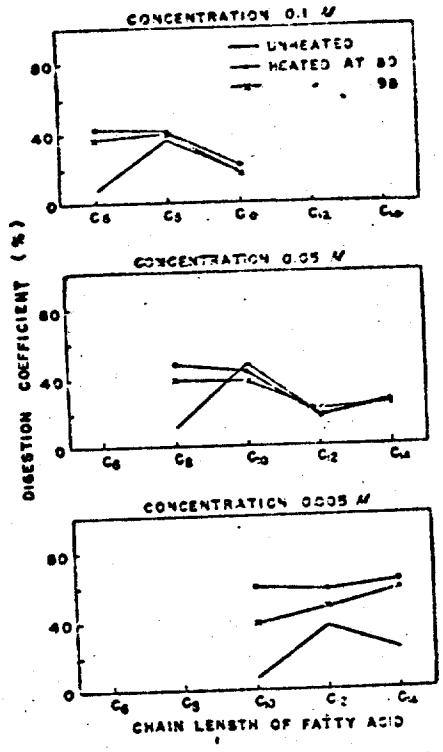

FIG. 1. Effect of fatty acid added at various concentrations on the digestion of ovalbumin by trypsin. Digestion time: 20 minutes.

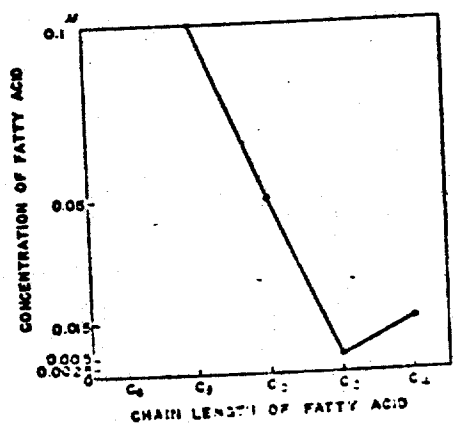

FIG. 2. Concentration of fatty acids at which the digestibility of native ovalbumin by trypsin exceeds 35 per cent. Digestion time: 20 minutes.

because the laevo-rotatory power of protein solution usually increases with its denaturation.

(*A*) *Samples:*

Native Crystalline Ovalbumin (*Previously described*)—1 per cent ovalbumin phosphate buffer solution (0.1 *M*, pH 7.8).

Fatty Acid Added Ovalbumin—One ml of 2 per cent ovalbumin phosphate buffer solution (0.2 *M*, pH 7.8) was added by proper quantities of fatty acid and 1 ml. of water to give final concentrations of 0.0025 *M*, 0.005 *M*, 0.015 *M* and 0.1 *M* respectively.

(*B*) *Experiments:*

The specific rotation of native ovalbumin (Sample a) was determined in a 5-cm. tube with Lippich's polarimeter at 25°. The fatty acid was added to ovalbumin solution (Sample b), the mixture was incubated 20 minutes at 37° followed by the centrifugation (12,000 r.p.m.) to remove the resulted turbidity. No detectable loss of nitrogen compounds by centrifugation was observed. The specific rotation was determined in the same way as above.

(*C*) *Results:*

It was shown that C_8 fatty acid did not cause any change in the optical rotation of ovalbumin solution even at the concentration of 0.05 *M*, but the rotation increased to laevo at 0.1 *M*. The longer fatty acids caused this

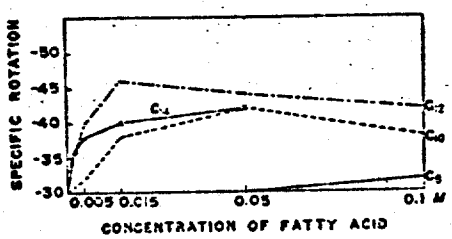

FIG. 3. Change in the optical rotation of ovalbumin by the addition of various fatty acids.

effect at lower concentrations, and C_{12} acid had the strongest effect at the concentration of 0.015 *M*.

DISCUSSION

The results obtained in the experiment I indicated that the addition of fatty acids to ovalbumin solution gave a change like the denaturation by heating, that is, an increase of digestibility by trypsin.

Concerning the effect of surface-active substances, it is reported by P.D. Boyer *et al.* (5) that denaturation of serum albumin is inhibited strongly by salts of fatty acids, especially by the acids whose carbon chain consists from 7 or 8 to 12 carbons. Also T. Sato recently reported that both the substrate (*i.e.*, hemoglobin, casein) and trypsin are denatured by sodium laurylsulfate (6). Considering these facts, it is apparent that ovalbumin is denatured by fatty acids and becomes more digestible by trypsin. This property of ovalbumin is entirely different from that reported on serum albumin (6). The optimum concentration of fatty acids at which tryptic

digestion of ovalbumin is distinctly promoted without heating differs from fatty acid to fatty acid. C_{12} acid had the strongest action among those tested and other fatty acids showed less effect. This is in accord with the fact that the denaturing action of alkylsulfate on the protein is most strong in C_{12} acid (*7*). The tryptic digestion of ovalbumin decreased above a certain concentration of added fatty acid, this might be explained by the assumption that fatty acids denaturate not only ovalbumin, but also trypsin itself as indicated in Sato's experiments. As the experiment II indicates, the optical rotation of ovalbumin solution is changeable with the addition of fatty acid, and the effect has almost the same tendency as on the tryptic digestion, that is, C_{12} acid has the strongest power at the lower concentration among the tested C_5 to C_{11} acids. It is generally believed that the change of optical rotation of the protein solution is caused by the denaturation, (of protein,) for instance by the unfolding of protein molecule (*8*). The results in the experiment I indicates that the denaturations by heating and by added fatty acids are probably the same kind of modification of proteins at least in a sense that both preparations are susceptible to tryptic digestion.

SUMMARY

1. C_5 to C_{11}-fatty acids promote the tryptic digestion of ovalbumin, but C_5-fatty acid does not at concentrations 0.1–0.4 *M*.

2. Ovalbumin becomes susceptible to tryptic digestion by the addition of fatty acids nearly as much as by heat denaturation.

3. At higher concentration of fatty acids the tryptic digestion decreased for both heated and unheated ovalbumin.

4. The optimum concentrations of fatty acids for tryptic digestion of ovalbumin decrease with the increase of the carbon chain.

5. The laevo-rotatory power of ovalbumin solution increases by the addition of fatty acid. The effect differs from fatty acid to fatty acid.

6. The greatest change of optical rotation is obtained by C_{12} acid at 0.015 *M* concentrations, indicating that C_{12} acid is most effective in a modification of protein structure. This fits in with the observation by tryptic digestion.

The author wishes to express her sincere thanks to Prof. S. Akabori and Assist. Prof. B. Maruo for their kind guidance and encouragement throughout this work.

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Inhibition of Glycolytic Enzymes of Normal and Neoplastic Liver by Free Fatty Acids

By M. A. Lea and G. Weber. (*Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana 46207, U.S.A.*)

Studies on normal rat liver showed an inhibition of key glycolytic enzymes by free fatty acids (Weber, Convery, Lea & Stamm, 1966; Weber, Lea, Convery & Stamm, 1967). The present work provides evidence that glycolytic enzymes of hepatomas are also subject to such inhibition.

Supernatant fractions of rat liver or hepatomas were preincubated for 10min. at 37° with octanoate or laurate. The activity of glycolytic enzymes and lactate production were then measured in the presence of added cofactors and substrate.

In hepatoma 3924-A a 50% inhibition of hexokinase activity was observed with 1·6mM-octanoate and 0·05mM-laurate. The greater effect of the longer-chain fatty acid was similar to that found in the normal liver, where concentrations of 2·9mM-octanoate and 0·08mM-laurate were required for 50% inhibition of hexokinase. Glucokinase activity was not detectable in the 3924-A tumour but in the normal liver a 50% inhibition was observed with 0·4mM-octanoate and 0·04mM-laurate. That the inhibitory effect of free fatty acids is not restricted to the liver is indicated by the action of octanoate on hexokinase activity of kidney cortex and 9789-K kidney tumour. A 50% inhibition was seen with 1·7mM-octanoate for the kidney cortex and 2·3mM-octanoate for the renal tumour.

The action of fatty acids on glycolytic enzymes other than those phosphorylating glucose was shown by the inhibition by laurate of phosphofructokinase activity in hepatomas 7800 and 3924-A. In addition, lactate production was inhibited with glucose or glucose 6-phosphate as substrate.

The inhibition of glycolytic enzymes by fatty acids may provide a mechanism for inhibitions of tumour growth observed with these compounds (Tolnai & Morgan, 1961).

The tumour-bearing rats were provided by Dr H. P. Morris of the National Cancer Institute, Bethesda, Md., U.S.A. The excellent technical assistance of Miss P. A. Jenkins and Miss R. A. Ross is acknowledged. This work was supported by grants from the U.S. Public Health Service CA-05034, The American Cancer Society and the Damon Runyon Fund.

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Inhibition of Liver Microsomal Inorganic Pyrophosphate–Glucose Phosphotransferase and Glucose 6-Phosphate Phosphohydrolase Activities by Metal-binding Agents

By R. C. Nordlie and P. T. Johns (introduced by W. J. Whelan). (*Department of Biochemistry, University of North Dakota Medical School, Grand Forks, North Dakota, U.S.A.*)

Citrate recently was found to inhibit both of the above activities of glucose 6-phosphatase, possibly by binding to enzyme-bound metal at the active site (Nordlie & Lygre, 1966). The effects of a variety of metal-binding agents on both activities have been studied to further explore the possibility that this catalyst is a metalloenzyme. Inhibition by chelating agents was investigated both by the classical kinetic approach (Dixon & Webb, 1964) and by pre-incubation techniques. The latter studies were carried out by pre-incubating the enzyme with inhibitors at pH 8·0 for 1hr. at 23°, and then assaying activities at pH 6·0. The following concentrations of metal-binding agents in pre-incubation mixtures produced 50% inhibition of phosphatase and phosphotransferase activities, respectively: 1,10-phenanthroline, 3 and 5mM; diethyldithiocarbamate, 0·70 and 0·73mM; and azide, 1·4 and 0·9M. Conventional studies of inhibition kinetics were conducted at pH 6·0 with azide, oxalate, 1,10-phenanthroline, and cyanide. K_i values determined with both activities agreed well for each inhibitor studied. Kinetically, inhibition was non-competitive with respect to glucose and competitive with respect to both inorganic pyrophosphate and glucose 6-phosphate. It thus appears that protein-bound metal may be involved in the binding of phosphate substrates to the enzyme.

Although inhibitions observed with *p*-chloromercuriphenyl sulphonate, *N*-ethylmaleimide, and iodoacetate suggested the involvement of protein thiol groups in the catalytic process, L-cysteine and dithiothreitol inhibited rather than stimulated activities. Inhibition by cysteine was additive with that by iodoacetate. *o*-Tyrosine was found to inhibit glucose 6-phosphatase activity in a manner additive with inhibition by *o*-hydroxyphenyl acetate rather than to reverse inhibition by the latter compound as previously reported (Feuer, Goldberg & Gibson, 1964). *o*-Tyrosine stabilized the enzyme against loss of activity by dialysis or

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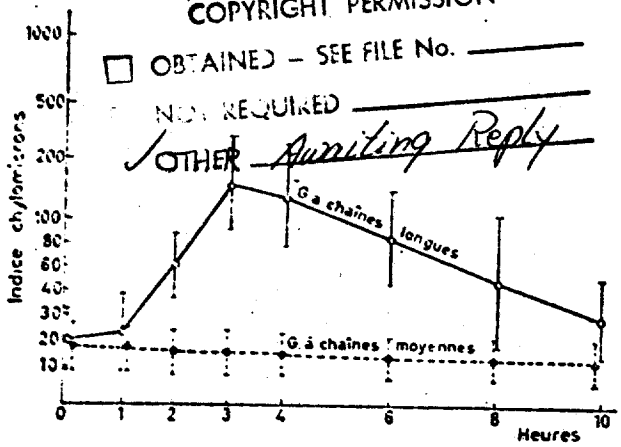

Fig. 4. Courbes d'hyperlipémie (indice-chylomicrons) de 10 sujets normaux après ingestion de 100 g d'huile d'arachide ou d'olive émulsionnée (graisse à chaînes longues) ou de 100 g de graisse synthétique émulsionnée (graisse à chaînes moyennes).

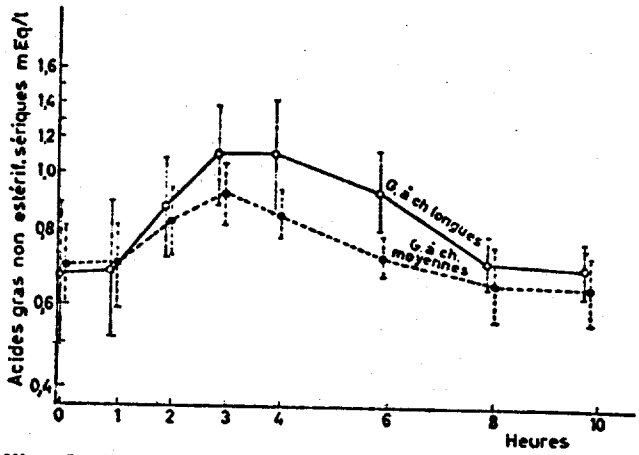

Fig. 5. Variations des acides gras non estérifiés sériques après ingestion de 100 g d'huile d'arachide ou de 100 g de graisse à chaînes moyennes (5 sujets).

Chez un sujet atteint d'une *chylurie* due à une fistule entre le système lymphatique et le bassinet gauche, un régime à base de TCM a arrêté l'élimination de chylomicrons urinaires en quelques jours, mais la chylurie réapparaît dès que le sujet est placé à un régime hypergras, à TCL.

Enfin plusieurs *hyperlipémiques* ont été placés pendant plusieurs jours, plusieurs semaines ou plusieurs mois à un régime contenant entre 60 et 100 g de TCM. Leur lipémie est revenue dans tous les cas à la normale, sauf chez un sujet atteint d'une très forte hyperlipémie (120 g ‰) à sérum lactescent, induite par les graisses et les glucides. Chez ce malade, le régime à base de TCM a ramené la lipémie à 40 g ‰, minimum qui n'a jamais pu être dépassé.

Les graisses à acides gras moyens présentent encore d'autres applications diététiques puisque facilement absorbées sans produire de chylomicrons, elles peuvent couvrir les besoins énergétiques, comme tous autres lipides sans en présenter certains inconvénients.

III — Travaux effectués au Centre de Recherche sur la cellule normale et cancéreuse, Villejuif

E. LE BRETON et P. LEMARCHAL :

Catabolisme de l'acide octanoïque par les mitochondries de foie de rat. Comparaison entre le foie normal et le foie stéatosique

On sait qu'un grand nombre de facteurs peuvent donner lieu chez l'homme et l'animal à une accumulation anormale de graisses dans le foie. Parmi ceux-ci, on trouve divers toxiques tels que les dérivés halogénés, le phosphore, l'exposition aux rayons X, ainsi que des facteurs hormonaux et nutritionnels, tels que l'ingestion de trop grandes quantités d'alcool.

La stéatose dont il est question ici sera du type nutritionnel, causée par l'administration d'un régime hypoprotéique et hyperlipidique. D'après E. Le Breton (1954), la carence protéique constitue un « facteur d'agression » susceptible de déclencher une hypersécrétion d'hormone surrénalienne (adrénaline), qui mobilise les graisses de réserve des dépôts (Clément, 1951). Les acides gras, véhiculés par le sang sous forme non estérifiée, sont captés par le foie et stockés dans cet organe sous forme de triglycérides. Si cette réserve lipidique n'est pas l'objet d'une utilisation accrue de la part de l'organe, soit par exportation de lipoprotéines vers le plasma, soit par β oxydation mitochondriale, la stéatose en résulte. On sait d'autre part que l'adjonction de vitamine B_{12} ou de choline dans le régime corrige cette accumulation des triglycérides hépatiques, par un mécanisme non encore complètement élucidé (Mazzei, 1967), d'où le nom de « lipotropes » qui leur a été donné.

Il a donc paru intéressant d'examiner l'un des mécanismes fondamentaux de l'utilisation des triglycérides hépatiques, à savoir le catabolisme mitochondrial *in vitro* des acides gras, comparativement chez des rats normaux ou présentant une stéatose hépatique nutritionnelle.

L'acide octanoïque a été choisi comme substrat, pour des raisons déjà exposées (Mazzei, 1967). L'une des raisons invoquées est l'effet destructeur des acides gras libres à longue chaîne et de leurs thioesters sur les membranes, effet d'autant moins prononcé que l'acide gras est plus court.

D'après des recherches antérieures (Lévy, 1951; Lévy et Legrand, 1953; Artom, 1953; Toury et Lévy, 1967), l'activité des systèmes d'oxydation de l'acide octanoïque mesurée *in vitro* subit une chute importante dans le foie stéatosique, comparé au foie normal. Cependant, la technique manométrique de Warburg utilisée ne permet pas de distinguer nettement l'activité octanoxydasique propre de la « respiration endogène » mitochondriale. On mesure en fait une consommation d'oxygène qui est la résultante du catabolisme de l'acide octanoïque et de l'oxydation de constituants biochimiques apportés avec l'extrait enzymatique lui-même. L'étude de l'oxydation de l'acide octanoïque a donc été faite à l'aide d'une technique plus spécifique en utilisant un substrat marqué par le ^{14}C. Ce sont les résultats de ces recherches qui sont exposés ici.

1. Matériel et méthodes

1) Animaux

Des rats Wistar (souche Commentry) reçoivent dès le sevrage et pendant un mois un régime standard équilibré. Ils sont ensuite divisés en deux groupes, soumis respectivement aux deux régimes suivants : stéatogène (M_2) et témoin équilibré (M_6), décrits sur le tableau I.

2) Incubations

Après un à cinq mois de régime, deux animaux (un de chaque groupe) sont sacrifiés. Une partie du foie est réservée au dosage des lipides. Sur une deuxième fraction, les mitochondries sont préparées suivant Schneider et Hogeboom et mises à incuber dans des conditions voisines de celles utilisées par Lehninger (1948) et par les différents auteurs précités (tableau II). Un marqueur radioactif en $^{8}C(CH_3)$ ou en ^{1}C (COOH) est ajouté au milieu. L'incubation est effectuée dans un tube de centrifugeuse bouché et muni de deux tubulures permettant ultérieurement de récupérer le gaz carbonique par barbotage d'azote.

Dans les conditions d'incubation précédentes, d'après Lehninger et en accord avec nos résultats, l'octanoate est transformé en acétoacétate suivant le schéma :

$$\text{octanoate} + 3O_2 \rightarrow 2 \text{ acétoacétate} + 2H_2O \quad (1)$$

$$C_7H_{15}COOH + 3O_2 \rightarrow 2CH_3 - CO - CH_2 - COOH + 2H_2O.$$

La réaction (1) suit en fonction du temps une loi de linéarité.

Nous avons observé qu'une quantité négligeable de $^{14}CO_2$ est dégagée au cours de la réaction, bien qu'il soit possible, en particulier en augmentant considérablement le rapport $\frac{N \text{ mitochondrial}}{\text{substrat}}$, d'obtenir une oxydation plus complète jusqu'à $^{14}CO_2$.

Pour chaque préparation mitochondriale, deux incubations sont effectuées, avec des mitochondries correspondant respectivement à 0,1 g et 0,2 g de foie.

TABLEAU I

Composition des régimes

	Régime M_2 (stéatogène)	Régime M_6 (témoin)
	(%)	(%)
Caséine dévitaminée	8	22
Saccharose	62	58
Saindoux	20	10
Mélange salin	5	5
Mélange vitaminique n° 4 (O choline OB_{12})	2,5	
Mélange vitaminique n° 2 (+ choline + B_{12})		2,5
Agar Agar	2,5	2,5

TABLEAU II

Milieu d'incubation

	µmoles
Tampon phosphate pH 7,4	40
Cl_2Mg	24
ClK	200
ATP	4
Cyt c	0,04
Octanoate de sodium non radioactif	1,6
Octanoate 8 ^{14}C ou 1 ^{14}C	0,02 à 0,05
Mitochondries, quantité correspondant à 0,1 ou 0,2 g de foie initial (1), en suspension dans ClK 11,5 ‰ (1 ml/g de foie).	

250 000 à 615 000 dpm au total dans chaque essai.
Incubation 30 mn à 28 ou 30° avec agitation.

(1) Deux incubations par essai.

3) Mesure de l'activité octanoxydasique

La réaction est arrêtée par addition de 0,5 ml d'acide chlorhydrique normal; le gaz carbonique dégagé est chassé par barbotage d'azote (10 ml/mn), pendant 15 mn à 0°.

On ajoute ensuite au milieu 10,5 ml d'acide trichloracétique à 10 % et 10 ml du mélange éther de pétrole-benzène 1-1 (V/V). Après agitation et centrifugation, il se forme trois phases :

— le précipité protéique;
— la phase aqueuse, contenant les corps cétoniques;
— la phase éthérée, contenant l'acide octanoïque restant après réaction.

Cette dernière est prélevée, et l'on extrait la phase aqueuse encore deux fois par le mélange éther de pétrole-benzène. La phase aqueuse est ensuite débarrassée des dernières traces de solvants organiques par balayage d'azote. Elle est ensuite prélevée, traitée par le bichromate et soumise à distillation selon Weischelbaum et Somogyi (distillation des quatre cinquièmes du volume total soumis à distillation). Nous savons que le bichromate oxyde l'acide β hydroxybutyrique éventuellement présent en acide acétoacétique, et que celui-ci est décarboxylé pour former de l'acétone :

$$CH_3 - CO - CH_2 - COOH \rightarrow CH_3 - CO - CH_3 + CO_2 \quad (2)$$

L'acétone formée est récupérée quantitativement dans le distillat; elle correspond donc à la totalité des corps cétoniques formés au cours de l'incubation. Le distillat est amené à volume connu, ainsi que l'ensemble des phases éthérées provenant de l'opération précédente. Des parties aliquotes sont prélevées pour le comptage de la radioactivité par scintillation liquide.

L'azote mitochondrial est dosé par la méthode de Kjeldahl ou de Nessler.

2. Résultats

1) Mise en évidence de la stéatose

Sur 6 couples de rats étudiés, 5 ont été trouvés porteurs d'un foie stéatosique ainsi qu'il résulte des teneurs en lipides (tableau III) chez certains; l'accumulation de lipides est considérable (surcharge égale à 6 fois la quantité normalement présente), dépendant du temps de régime. Une étude en microscopie électronique a montré la présence de gouttelettes lipidiques réparties au sein du cytoplasme, et même du nucléoplasme, accompagnant une hypertrophie du système de Golgi.

2) Activité octanoxydasique

Le tableau III résume les résultats obtenus, en indiquant, pour chaque préparation mitochondriale, en p. 100 de la radioactivité initiale, la radioactivité restant dans la phase éthérée (octanoate), celle apparue dans la phase aqueuse (corps cétoniques) et celle du $^{14}CO_2$.

On notera, dans le cas d'un substrat $1^{14}C$, que la radioactivité de l'acétone n'a pas été recherchée, puisque la décarboxylation même de l'acétoacétate élimine la radioactivité correspondante sous forme de $^{14}CO_2$.

On peut constater tout d'abord que la radioactivité du gaz carbonique dégagé est négligeable, que l'octanoate initial soit marqué en position 8 ou 1. Les enzymes du cycle de Krebs n'interviennent donc pas dans les conditions de milieu choisies. Dans le cas d'un substrat $8^{14}C$, toute la radioactivité de l'octanoate transformé est retrouvée dans l'acétone du distillat, c'est-à-dire dans les corps cétoniques. Ces faits sont conformes au schéma de la réaction 1, mais n'en constituent pas une preuve absolue, la radioactivité des corps cétoniques n'ayant pu être mesurée dans le cas d'un substrat marqué en position 1.

TABLEAU III

Radioactivités après incubation

(en % de la radioactivité initiale en 30 mn et pour 0,1 g de foie)

N° exp.	Semaines de régime	Régime	Surcharge lipidique mg/g foie	Substrat	Disparue dans l'octanoate	Apparue dans l'acétone	Apparue dans CO_2
1	6	M_2 ...	124	$8^{14}C$	32	30	0,6
		M_6 ...	0	$8^{14}C$	31	27	0,3
2 (28°)	6	M_2 ...	39	$8^{14}C$	21	18	0,8
		M_6 ...	0	$8^{14}C$	22	17	0,6
3	6	M_2 ...	5	$8^{14}C$	35	26	2,5
		M_6 ...	0	$8^{14}C$	36	25	0,5
4	8	M_2 ...	177	$8^{14}C$	15	13	
		M_6 ...	0	$8^{14}C$	17	14	
5	8	M_2 ...	402	$1^{14}C$	26		1,3
		M_6 ...	0	$1^{14}C$	27		0,8
6	22	M_2 ...	370	$1^{14}C$	18		
		M_6 ...	0	$1^{14}C$	48		

Il apparaît clairement par contre, si l'on compare les résultats obtenus avec les foies stéatosiques d'une part, les foies normaux de l'autre, que la vitesse de disparition de l'acide octanoïque, ramenée au gramme de foie frais initial, est la même dans les deux cas.

La seule exception concerne le rat n° 6 (celui qui a été maintenu le plus longtemps au régime stéatogène). En ramenant la vitesse de disparition de l'octanoate au milligramme d'azote mitochondrial, on voit qu'elle est même sensiblement supérieure dans les foies stéatosiques (tableau IV). Ceux-ci, du fait de leur surcharge en graisses, ont en effet une teneur en azote mitochondrial par gramme de foie frais généralement inférieure à celle des foies normaux, ainsi qu'il apparaît sur le tableau IV et en accord avec des résultats antérieurs (Mazzei, 1967).

TABLEAU IV

Vitesses de disparition de l'octanoate

N° exp.	Semaines de régime	Régime	Surcharge lipidique mg/g foie	Azote mitochondrial mg/g foie	Vitesse *(a)*	Vitesse *(b)*
1	6	M_2	124	3,5	170	49
		M_6	0	4,0	165	41
2	6	M_2	39	2,3	112	49
		M_6	0	3,4	117	34
3	6	M_2	5	4,2	186	44
		M_6	0	4,8	192	40
4	8	M_2	177	3,64	80	22
		M_6	0	4,76	91	19
5	8	M_2	402	3,40	138	41
		M_6	0	4,02	144	36
6	22	M_2	370	1,96	99	50,9
		M_6	0	4,02	259	64

(a) En m/μ moles par minute et par g de foie.
(b) En m/μ moles par minute et par mg d'azote mitochondrial.
Chaque valeur est la moyenne de deux mesures.

3. Discussion et conclusion

L'exposé de nos résultats appelle une première remarque. Nous avons admis dans un premier temps que la réaction enzymatique principale intervenant lors des incubations est la transformation de l'octanoate en acétoacétate (ou β hydroxybutyrate). Nous nous sommes appuyés pour cela sur les recherches de Lehninger, et sur l'absence de $^{14}CO_2$ que nous avons nous-mêmes observée. Une confirmation de ce fait par une analyse de la phase éthérée ainsi que de la phase aqueuse par des techniques spécifiques de dosage des acides gras et cétoniques serait en toute rigueur nécessaire. Cette réserve étant formulée, ces résultats sont interprétés de la façon suivante : bien que la stéatose hépatique de nos rats soit très importante (surcharge atteignant 400 mg par gramme de foie), l'activité octanoxydasique de leurs mitochondries, exprimée en terme d'octanoate disparu, par unité de temps et par milligramme d'azote mitochondrial, est supérieure ou égale à celle des mitochondries hépatiques de rats normaux prises dans les mêmes conditions.

Si l'on constate, comme l'ont fait divers auteurs, une diminution de la respiration mitochondriale, en cas de stéatose nutritionnelle, exprimée en terme de consommation d'oxygène, il n'est pas certain, d'après les résultats exposés ici, que cette diminution intéresse le catabolisme de l'acide octanoïque proprement dit. On peut envisager au contraire, au moins dans les premiers temps (un à deux mois de régime), que le jeune animal réagisse à la surcharge hépatique en graisses par une β oxydation légèrement accrue, insuffisante cependant pour compenser l'afflux des acides gras apportés par le courant sanguin. Si la situation de carence protéique se prolonge, on observe que cette surcharge s'accompagne d'une disparition presque totale des dépôts adipeux de réserve, et plus généralement d'un état physiopathologique extrêmement défavorable (amaigrissement sévère, pelage hérissé). Il est possible que, dans ce cas, le métabolisme mitochondrial hépatique se trouve profondément perturbé, et en particulier l'activité β oxydante. Il ne s'agirait cependant là que d'une situation tout à fait exceptionnelle, celle de jeunes animaux en croissance et soumis pendant un temps prolongé à un régime très déséquilibré.

L'impossibilité où nous avons été d'obtenir même un début de stéatose, avec ce même régime mais chez des rats ayant 8 mois ou plus, constitue une bonne illustration de cette hypothèse.

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Discussion sur les acides gras courts et moyens

R. F. — Après lecture du rapport de M. Clément et coll., on peut se poser la question de savoir si les acides gras courts sont bien utilisés par l'organisme comme les autres acides gras classiques, étant donné qu'ils se présentent eux aussi sous forme de glycérides généralement non homogènes.

G. C. — Ces acides gras, sous cette forme, sont tout à fait bien utilisés par l'organisme, aussi bien par l'organisme total comme l'ont montré beaucoup d'expériences antérieures et plus récemment celles du Pr Métais, que ceux qui sont introduits par voie intraveineuse. M. Métais a montré que ces acides gras sont très rapidement oxydés. Nous trouvons par des perfusions hépatiques ou pulmonaires une oxydation, une métabolisation très rapide. On a même l'impression que, lorsqu'on administre des glycérides homogènes d'acides gras courts, comme la trioctanoïne ou les mélanges des glycérides en $C_6 - C_8$, que l'oxydation est extrêmement rapide, beaucoup plus que celle de l'huile de coprah par exemple.

R. F. — Est-ce que vous pensez que les glycérides d'acides gras courts et moyens ont un intérêt particulier ?

G. C. — La réponse paraît bien être oui. Les diététiciens ont trouvé des applications pour certains malades.

R. F. — Et pour l'homme normal ?

G. C. — Ces glycérides peuvent toujours couvrir au moins les besoins énergétiques.

R. F. — Mais ont-ils un intérêt particulier ?

E. B. — Ce sont des acides gras qui sont très vite oxydés lorsque l'animal a froid.

G. C. — ... et qui ne sont pas stockés. L'acide laurique est stocké en partie, mais les acides gras à chaîne plus courte ne se retrouvent qu'à l'état de trace dans les tissus de réserve.

E. B. — Ces acides gras sont vraiment utilisés préférentiellement pour lutter contre le froid, ce qui est déjà intéressant.

J. T. — Si vous vous placez sur le plan de la nutrition humaine, ces acides gras prennent la place d'autre chose.

C'est comme l'alcool, dans une certaine mesure. On observe une oxydation préférentielle.

R. F. — Peut-on dire, par ailleurs, qu'ils peuvent avoir des inconvénients spécifiques ?

G. C. — Pour le moment, je ne peux pas vous répondre.

J. T. — Nous sommes ici dans la médecine. Vraisemblablement ces glycérides qui sont absorbés rapidement doivent être agréables à consommer.

R. F. — C'est exact.

J. T. — Ces produits ont l'inconvénient de ceux qui sont oxydés facilement. Le risque de surcharge calorique est facile.

R. F. — Ce sont des produits qui ont les inconvénients de leurs qualités.

G. C. — Ils ne donnent pas lieu à la formation de chylomicrons, ce qui peut être intéressant dans certains cas.

J. T. — A condition bien entendu de ne pas créer de surcharge calorique.

R. F. — Une bonne alimentation doit comporter un mélange d'acides gras. Quel est le pourcentage acceptable d'acides gras courts dans un bon régime ?

E. B. — Leur absorption facile ne permet pas de fixer une limite. Il ne s'agit que d'une source de calories pour l'organisme, ce n'est pas autre chose.

R. F. — Peut-on dire que l'absence de ces acides gras courts et moyens dans l'alimentation semble être sans inconvénients (des populations entières en sont dépourvues), mais que, au contraire, leur utilisation, dans une mesure raisonnable, est sans inconvénient ?

E. B. — C'est un nutriment qui, à mon sens, n'a aucun caractère de nécessité, mais ne présente aucun inconvénient (1).

R. J. — Et qui peut être une arme diététique dans certains cas spéciaux.

R. F. — Je voudrais faire remarquer que la quantité d'acides gras courts et moyens dans la nature est limitée et que le risque d'en abuser se trouve ainsi réduit, du moins en moyenne.

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(1) Des expériences de E. Le Breton et P. Lemarchal (en cours de publication) montrent que même dans le cas d'une *forte stéatose hépatique*, l'acide octanoïque est oxydé avec une vitesse normale, à condition que le régime contienne assez de protéines (6 à 8 %) pour permettre la synthèse des enzymes impliqués. Ce résultat s'oppose à des faits antérieurs et renforce l'idée d'une bonne utilisation de l'acide octanoïque par les mitochondries et pas seulement pour fournir de la chaleur.

(approximately +16%) represents abnormal thyroid enlargement or that the mean thyroid size is greater by [illegible] Nithsdale children.

[illegible]graphical variations, both national and local, in goitre [illegible]idence in children and adults in the British Isles have been a regular feature of previous careful surveys (Stocks 1928, Erskine 1933, O'Shea 1946, Murray et al. 1948, Hughes et al. 1959, Trotter et al. 1962, Kilpatrick et al. 1963) and the higher prevalences have almost invariably been found in rural areas where other thyroid abnormalities may have a persistently high incidence. Wilkes' (1966) survey in Derbyshire emphasises this.

More than 100 years ago Mitchell (1862) described goitre in the Nithsdale Valley and the condition seemed to be common enough to merit the term "Nith neck". Local physicians still refer to this term in describing thyroid enlargement in this area (Laurie and Drainer 1967). Other small geographical pockets of endemic goitre in Scotland have been described in the last century (Sloan 1883, McKenzie 1899, Ogilvy 1911, McKay 1914–17).

The ætiology of the goitres found in the present study is not known. In a detailed study of 172 pairs of twins, aged from 12 to 70 years (mean, 21·7 years) from the Glasgow area, the ætiological importance of genetic predisposition, autoimmunity, and iodine deficiency as causes of simple goitre were evaluated. None of these factors were found adequately to correlate with the presence of goitre (McGirr 1966, Greig et al. 1967). The only positive conclusion derived from this study was that goitre in young people is related to a non-genetic female endocrine influence. Our results are consistent with this conclusion, goitre being positively related to the age range 11–15 years and to the female sex, in Glasgow and Dumfries where the [illegible]ce of goitre was relatively low.

[illegible]ctively greater prevalence of goitre in pubertal females than in pubertal males is likely to be relevant to the persistence of goitre in adulthood, in which the female/male ratio is high. Whether during and after puberty the female endocrine influence is progoitrogenic or the male endocrine influence antigoitrogenic has, however, yet to be determined. The difference is so striking as to make it unlikely that differences in thyroid-hormone requirements and in iodine requirements alone would account for it.

The higher prevalence of goitre in Nithsdale is not associated with a difference in prevalence among females and males up to the age of 15 years. This, together with the possibility that goitre has been more prevalent in this area for more than 100 years notwithstanding population migration, suggests that an extrinsic factor is mainly responsible for the thyroid enlargements in this rural area. The lack of a preponderance of goitre in the Nithsdale female children may favour the hypothesis that any intrinsic goitrogenic endocrine influence present in adolescence has been preceded by and relegated to a subsidiary role because of the life-long effects of an extrinsic or environmental factor.

Comparative studies are now being made to determine whether this excess of goitre prevalence in Nithsdale is due to correctable factors such as iodine-deficiency state (Crooks et al. 1964, Wayne et al. 1964), or of goitrogenic substances in the flora, milk, and water of the region (Clements 1960, Peltola 1960, Kilpatrick and Wilson 1964).

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EFFECTS OF OCTANOIC ACID ON SERUM-LEVELS OF FREE FATTY ACIDS, INSULIN, AND GLUCOSE IN PATIENTS WITH CIRRHOSIS AND IN HEALTHY VOLUNTEERS

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Summary The effects of intraduodenal infusions of glucose and octanoic acid on the serum-levels of glucose, free fatty acids (F.F.A.), and insulin have been determined in four healthy volunteers and in five patients with cirrhosis. Serum-insulin levels rose in both groups during glucose infusions but the rise was significantly greater when octanoic acid was added. This effect did not seem to be mediated by the serum-glucose because the addition of octanoic acid did not affect this level. Serum-F.F.A. levels fell in both groups but the inclusion of octanoic acid had no effect on the rate of fall of serum-F.F.A.

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Cap. Acid

Introduction

IMPAIRED glucose tolerance with elevated fatty-acid levels has been described in patients with obesity (Karam et al. 1965), maturity-onset diabetes (Laurell 1956, Bierman et al. 1957, Randle et al. 1963), acromegaly (Karam et al. 1965), and cirrhosis (Reinberg and Lipson 1950, Dole 1956, Stormont et al. 1961). Randle et al. (1963) suggested that the elevated serum-free-fatty-acid (F.F.A.) levels common to all of these diseases may contribute in part to the reduction in glucose tolerance observed. Furthermore, glucose tolerance has been shown to be impaired in healthy individuals when serum-F.F.A. has been raised by a high fat intake followed by intravenous heparin (Schalch and Kipnis 1965). We have investigated hepatic glucose production as a factor in regulating glucose tolerance in the presence of a F.F.A. flux. A rapid increase of fatty acids in the portal circulation was obtained by adding a medium-chain fatty acid (M.C.F.A.) to a duodenal infusion of glucose. M.C.F.A.s were used because they are transported from the gut by the portal venous system (Kiyasu et al. 1952, Playoust and Isselbacher 1964, Greenberger et al. 1966), in contrast to long-chain fatty acids which are absorbed via the lymphatics. Thus the enteral administration of M.C.F.A. gives rise to the portal perfusion of the liver with these fatty acids and results in acute quantitative as well as qualitative changes of the fatty acids in the portal circulation.

Volunteers, Patients, and Methods

Volunteers and Patients

Four healthy male volunteers, aged between 20 and 35, without any family history of diabetes mellitus were used as controls. Diabetes mellitus was excluded by performing an oral glucose tolerance test (100 g.).

Five patients with moderately severe cirrhosis of the liver were investigated. None had a clinical history of diabetes or of glycosuria (table 1). We included patients with cirrhosis because we had found significantly higher serum-octanoic-acid levels after intraduodenally infused octanoic acid in such patients than in non-cirrhotic patients (Linscheer, Patterson, Moore, Clermont, Robins, and Chalmers 1966).

TABLE I—CLINICAL DATA ON THE PATIENTS WITH CIRRHOSIS

No.	Age	Sex	Ascites	Varices	Alcohol intake	Serum-levels of: Total bilirubin (mg. per 100 ml.)	Alkaline phosphatase B.L. units	Albumin/globulin (g. per 100 ml.)	Prothrombin-time (%)
1	44	M	+	+	+	0·3	4·0	3·7/2·0	64
2	67	F	+	+	+	0·7	6·5	3·4/3·4	50
3	51	M	+	−	+	1·4	5·7	2·6/3·9	29
4	65	M	−	+	+	1·0	3·1	3·3/4·8	47
5*	48	M	−	−	+	0·8	7·0	2·8/3·3	8·2

* Laennec cirrhosis diagnosed at liver biopsy.
B.L. = Bessey-Lowry.

Experimental Design

Two glucose infusions were performed in all subjects. Octanoic acid was added to one of the two infusions in random order. Serum glucose, insulin, octanoic acid, and total F.F.A. were determined before, during, and after the infusion procedure. Octanoic acid alone was infused in two cirrhotic patients.

Technique of Duodenal Infusion

A one-lumen tube (diameter 6 mm.) was passed into the proximal duodenum and positioned under fluoroscopic control. After the tube was properly situated, a test solution was infused for 60 minutes at a constant rate of 6 ml. per minute by means of a Holter infusion pump. The order of the infusion was randomly selected and was performed on different days. At the end of the 60-minute infusion period the tube was removed. Blood-samples for the determination of serum-total-F.F.A., octanoic acid, glucose, and insulin were drawn before, at 10-minute intervals during the 60-minute infusion period, and during the first 2 hours after the removal of the tube (15-minute intervals during the first hour and 30-minute intervals during the second hour).

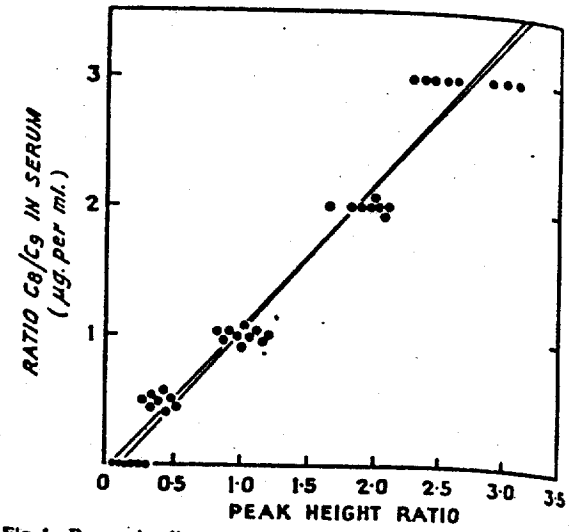

Fig. 1—Regression lines (x on y and y on x) for the ratio of octanoic-acid concentration (0–60 µg. per ml.) to 20 µg. nonanoic acid per ml. and the ratio of the peak heights on the gas-liquid chromatogram. $x = 0{\cdot}0874 + 0{\cdot}8724y$; $y = -0{\cdot}0613 + 1{\cdot}1135x$.

Infusate

Both test solutions contained glucose (138·9 mg. per ml.), sodium-taurocholate (20 mg. per ml.), and sodium chloride (8·5 mg. per ml.) in water; one solution also contained octanoic acid (10 mg. per ml.).

Analysis of Serum-total-F.F.A.

The Dole method was used when no M.C.F.A.s were present (glucose infusion without octanoic acid). A modification of the Dole method in combination with quantitative gas/liquid chromatography was used when octanoic acid was also present in serum.

Serum (1 ml.) was extracted by 5 ml. isopropanol/heptane/N sulphuric-acid, 4/10/1 (v/v). The heptane layer was then separated by the addition of water (2 ml.) and heptane (3 ml.). The upper phase (3 ml.) was titrated with 0·015 N sodium-hydroxide solution as described by Dole. The long-chain F.F.A. fraction of the serum was completely extracted by this procedure but only a partial extraction (58%) was obtained for octanoic acid. In the absence of octanoic acid a single standard, palmitic acid was used to express the long-chain F.F.A. in µEq. per litre. The serum-F.F.A. is then the product of the nominal value and the ratio of titration equivalents of unknown and palmitic-acid standards. This calculation is invalid if octanoic acid is also present in serum since this acid is incompletely extracted by this method.

The serum-octanoic-acid fraction was measured by quantitative gas/liquid chromatography. The octanoic acid determined by this method is expressed in µEq. per litre and converted into µEq. sodium hydroxide per litre, using the octanoic-acid standard. This is then subtracted from the total amount of sodium hydroxide used for the simultaneous titration of octanoic acid and long-chain F.F.A. extracted from serum (1 ml.). The difference is the long-chain F.F.A. fraction and can be expressed as µEq. per litre using palmitic acid as a standard. Thus the long-chain F.F.A. and octanoic-acid serum fractions are known, and the sum of the two fractions will give a value for the total serum-F.F.A.

Determination of Serum-octanoic-acid by Quantitative Gas/liquid Chromatography

As an internal standard, nonanoic acid (20 μg.) dissolved in acetone (0·04 ml.) was added to each ml. of serum. The serum (1 ml.) was extracted by the Dole procedure (see above). The internal standard allows for correction for incomplete extraction of octanoic acid by the Dole method. After separation of the heptane layer the upper phase (3 ml.) was divided into three portions each. Each portion was evaporated to dryness at 0 C in vacuo by lyophilisation. The dry fatty-acid samples were dissolved in carbon disulphide and a fraction (0·5–1·0 μl.) was used for injection into the gas chromatograph. Octanoic acid in serum was calculated by using the ratios of peak heights (C8/C9) and the ratios of C8 and C9 (C8/C9) in serum (fig. 1). Since C9 in serum is constant (20 μg. per ml.), concentration of C8 can be calculated from the equation:

$$y = 0{\cdot}0613 + 1{\cdot}1135x$$

$$y = \frac{\mu g.\ C8/ml.\ serum}{\mu g.\ C9/ml.\ serum} \text{ and } x = \frac{\text{peak height C8}}{\text{peak height C9}}$$

Standard conditions were vaporiser temperature 260°C, column temperature 180°C, detector temperature 230°C, nitrogen pressure 20 lb. per sq. in. Peaks were identified by retention-time and standards.

Insulin Measurements

Serum-insulin was measured by an immunoassay method (Soeldner and Slone 1965). To test a possible effect of octanoic acid on serum-insulin determinations, octanoic acid (20–200 μEq. per litre) was added to pooled serum, containing 44 μI.U. of insulin. No detectable effect of octanoic acid on the insulin measurements was observed.

Glucose Measurements

True serum-glucose was measured by the glucose-oxidase technique (Washko and Rice 1961).

Statistical Analysis

Since octanoic acid has a high absorption-rate and is metabolised immediately (Schwabe et al. 1964), a statistical analysis was performed on the glucose and insulin data obtained during the infusion period (0–60 minutes). The serum-F.F.A. levels decreased after 10 minutes and the regression lines were calculated for the period between 10 and 80 minutes. Regression lines representing the correlation between serum glucose, insulin, and total F.F.A. concentrations and time were expressed by the formula $y = a + bx$ ($r > 0{\cdot}9$ in all instances). The effect of octanoic acid was measured by determining differences between the slopes of the regression lines and the standard error of the differences.

Results

Serum-glucose Response

In all four volunteers a rise of approximately twice the fasting levels of glucose occurred during the duodenal infusion. A return to preinfusion levels of serum-glucose was reached 45 minutes after the tube had been removed. The addition of octanoic acid to the infusate did not result in significant differences in glucose levels (fig. 2, table II).

In four of the five cirrhotic patients, serum-glucose rose within 10 minutes of starting the infusion. Serum-glucose levels were still elevated 45 minutes after discontinuation of the infusion in all five patients. The addition of octanoic acid to the infusion did not change the serum-glucose concentrations (fig. 2).

Serum-insulin Response

All four volunteers had fasting serum-insulin levels between 2 and 24 μI.U. per ml. A rise in insulin levels concomitant with glucose changes occurred in all cases. In no instance did the serum-insulin exceed 150 μI.U. per ml. (fig. 2). A return to fasting levels of insulin occurred 45 minutes after removal of the duodenal tube in all cases. This corresponded to the return of normal glucose levels. The addition of octanoic acid to a second infusion of glucose resulted in significantly higher insulin levels ($P < 0{\cdot}01$).

Two of the five cirrhotic patients had elevated fasting serum-insulin levels. A rise in serum-insulin occurred in all patients. The maximum rise for each patient did not exceed 160 μI.U. per ml. Addition of octanoic acid to the infusion resulted in peak levels greater than 160 μI.U. per ml. in four of the five patients studied. As in the volunteers, the insulin levels were significantly higher ($P < 0{\cdot}01$)

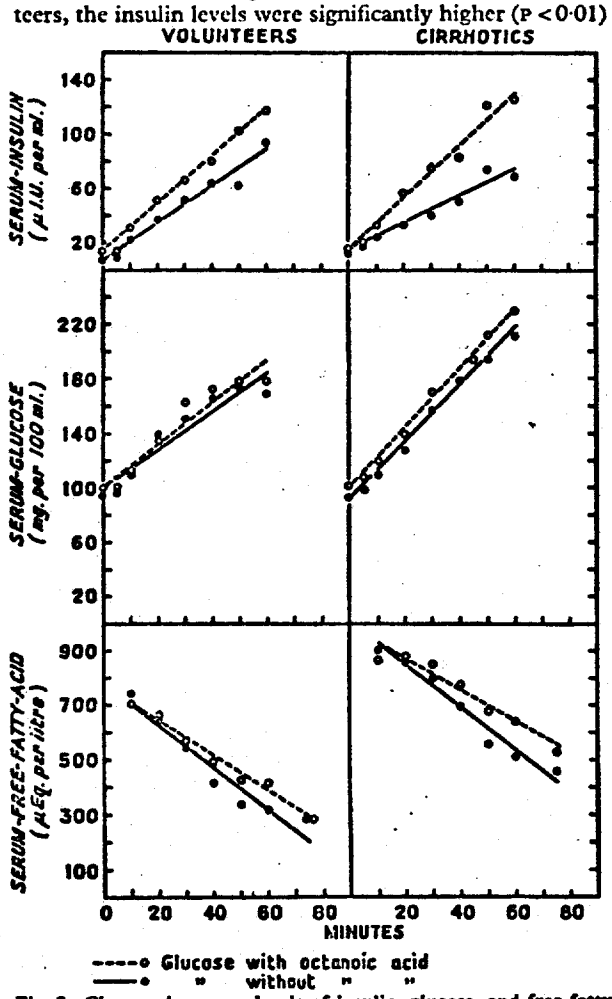

Fig. 2—Changes in serum levels of insulin, glucose, and free fatty acids in controls and in cirrhotic patients receiving glucose with or without octanoic acid.

INSULIN

Controls:
With octanoic acid: $y = 11{\cdot}54 + 1{\cdot}764x$; $r = 0{\cdot}997$.
Without octanoic acid: $y = 7{\cdot}65 + 1{\cdot}330x$; $r = 0{\cdot}981$.

Cirrhotic patients:
With octanoic acid: $y = 15{\cdot}86 + 1{\cdot}879x$; $r = 0{\cdot}962$.
Without octanoic acid: $y = 15{\cdot}72 + 0{\cdot}969x$; $r = 0{\cdot}971$.

GLUCOSE

Controls:
With octanoic acid: $y = 101{\cdot}51 + 1{\cdot}525x$; $r = 0{\cdot}964$.
Without octanoic acid: $y = 99{\cdot}27 + 1{\cdot}435x$; $r = 0{\cdot}959$.

Cirrhotic patients:
With octanoic acid: $y = 97{\cdot}96 + 2{\cdot}259x$; $r = 0{\cdot}996$.
Without octanoic acid: $y = 89{\cdot}07 + 2{\cdot}095x$; $r = 0{\cdot}997$.

FREE FATTY ACID

Controls:
With octanoic acid: $y = 760 - 6{\cdot}356x$; $r = 0{\cdot}992$.
Without octanoic acid: $y = 768 - 7{\cdot}410x$; $r = 0{\cdot}955$.

Cirrhotic patients:
With octanoic acid: $y = 974 - 5{\cdot}676x$; $r = 0{\cdot}979$.
Without octanoic acid: $y = 991 - 7{\cdot}600x$; $r = 0{\cdot}973$.

TABLE II—CHANGES IN SERUM GLUCOSE, INSULIN, TOTAL F.F.A., AND OCTANOIC ACID FROM FASTING LEVELS DURING INTRADUODENAL INFUSION OF GLUCOSE (A) AND GLUCOSE AND OCTANOIC ACID (B)

Serum-level	—	Values and differences at (min.):						
		5	10	20	30	40	50	60
Volunteers (4):								
Glucose (mg. per 100 ml.)	A	5	17	43	56	72	81	75
	B	2	14	36	63	73	79	80
	B–A	3	3	−7	+7	+1	−2	+5
Insulin (μI.U. per ml.)	A	2	14	29	43	56	54	85
	B	−4	13	32	47	61	84	98
	B–A	−2	−1	+3	+4	+5	+30	+13
Total F.F.A. (μEq. per l.)	A	+35	+74	−34	−129	−261	−333	−351
	B	+39	+53	+11	−80	−159	−223	−240
	B–A	+4	−21	+45	+49	+102	+110	+111
Octanoic acid (μEq. per l.).. ..	A	1	1	1	0	0	0	1
	B	+14	+58	+84	130	122	92	96
	B–A	+13	+57	+83	+130	+122	+92	+95
Cirrhotic patients (5):								
Glucose (mg. per 100 ml.)	A	4	15	33	63	85	101	118
	B	5	16	36	68	92	110	126
	B–A	+1	+1	+3	+5	+7	+9	+7
Insulin (μI.U. per ml.)	A	1	16	16	21	34	56	50
	B	5	17	40	58	66	115	110
	B–A	+4	+1	+24	+37	+32	+59	+60
Total F.F.A. (μEq. per l.)	A	50	29	−10	−74	−176	−318	−362
	B	17	24	31	3	−74	−169	−205
	B–A	−33	−5	+41	+77	+102	+149	+157
Octanoic acid (μEq. per l.).. ..	A	1	0	0	1	0	1	0
	B	16	65	145	169	176	193	198
	B–A	+15	+65	+145	+168	+176	+192	+198

when octanoic acid was demonstrable in the systemic circulation. As observed with glucose alone, a delayed return of insulin to preinfusion levels was noted (table II, fig. 2).

Serum-total-F.F.A.

Serum-total-F.F.A. levels fell in all volunteers during the glucose infusion (fig. 2, table II). The lowest level was reached between 15 and 30 minutes after the glucose infusion had stopped and when serum-glucose was receding from the maximal levels reached. 2 hours after ending the infusion the serum-total-F.F.A. had rebounded to fasting levels in all cases. The inclusion of octanoic acid had no significant effect on the rate of fall of the serum-F.F.A. ($P < 0.5$).

A fall in serum-total-F.F.A. level occurred in all cirrhotic patients during the glucose infusion. The lowest level was reached between 60 and 90 minutes after the termination of the glucose infusion. The addition of octanoic acid to the glucose infusion did not result in a significant difference ($0.10 > P > 0.05$) (fig. 2, table II).

Serum-octanoic-acid Response

No measurable levels of octanoic acid were detectable in the four volunteers during the glucose infusion. When octanoic acid was added to the glucose infusion all volunteers showed measurable levels of octanoic acid within 10 minutes of the beginning of the infusion. A maximum concentration was observed within 30 minutes and a return to fasting levels occurred within 45 to 60 minutes after discontinuation of the infusion procedure.

In all five cirrhotic patients no measurable levels of octanoic acid were detected during the glucose infusion alone. Addition of octanoic acid to the infusate resulted in detectable amounts of octanoic acid in the serum within 10 minutes of beginning the infusion. After discontinuation of the infusion, a return of fasting levels of octanoic acid was delayed as compared to the volunteers.

Octanoic Acid Infused Alone by Intraduodenal Tube

Two patients with cirrhosis were investigated. No change in glucose or insulin concentration was detected throughout the infusion. Serum-octanoic-acid levels were observed which were identical with those of cirrhotics given an infusion containing octanoic acid plus glucose.

Discussion

The addition of small amounts of octanoic acid to intraduodenal infusions of glucose (50 mg.) resulted in elevated levels of serum-immunoreactive-insulin as compared with intraduodenal glucose infusions alone. This was true of both healthy volunteers and patients with cirrhosis of the liver. This effect is probably not accounted for by a rise in serum-glucose, since no significant difference in glucose concentrations was observed. It is of interest to note that the serum-insulin rose only during the period of octanoic-acid absorption and it is conceivable that a causal relationship exists.

The finding that the serum-glucose levels remained unchanged during the paired infusion experiments suggests that octanoic acid did not alter the hepatic production of glucose.

Although the slopes of the regression lines of the fatty-acid levels were not significantly different, the fatty-acid levels were probably higher during the octanoic-acid infusion (fig. 2). These slight differences in F.F.A. levels may have been responsible for the observed increase of serum-insulin concentrations, although in the experiments described by Schalch and Kipnis (1965), the glucose tolerance and plasma-insulin levels were affected only by drastic increases in serum-F.F.A.

If the elevation of serum-insulin is not accounted for by the slight rise of F.F.A. during the octanoic-acid infusion, it is possible that octanoic acid exerted a direct stimulatory effect upon the pancreas, or alternatively stimulated the release of an unknown factor in the bowel

wall which then affected the release of pancreatic insulin. It is interesting that Horino et al. (1966) have observed a rise in serum-insulin in sheep in response to the intravenous infusion of short-chain fatty acids. This rise also occurred in the presence of unchanged serum-glucose levels.

It has been suggested that the formation of serum-ketoacids, after the administration of the M.C.F.A.s, are responsible for the stimulation of insulin secretion (T'Yangkul et al. 1965). We did not measure serum-ketoacids.

The cirrhotic patients showed significantly higher levels of serum-glucose, insulin, and F.F.A., but the two groups were not age-matched and as glucose tolerance decreases in the older age-groups no conclusions are drawn (Streeten et al. 1965).

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THERAPEUTIC HAZARDS OF PHENYLBUTAZONE AND OXYPHENBUTAZONE IN POLYMYALGIA RHEUMATICA

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POLYMYALGIA RHEUMATICA and temporal arteritis are accepted nowadays as different manifestations of a disseminated arteritis. In most cases of temporal arteritis the diagnosis is obvious at the first examination of the patient. In patients with primarily myalgic symptoms, however, the diagnosis is often obscure for a long time, and the selection of patients for treatment with corticosteroids—the only available treatment that can protect against ocular complications—is difficult. Visual loss is uncommon in myalgic patients, initially free from headache but, according to Olhagen (1963) and Balmforth (1964), this can happen. Thus, the observation of extracranial arteritis in myalgic patients must be of definite clinical importance; patients with suspected polymyalgia rheumatica should be instructed to report immediately to their doctor if they have thickening or pain in the distribution of the temporal artery. We have seen polymyalgic disorders develop into typical temporal arteritis in four patients undergoing therapy with phenylbutazone or oxyphenbutazone. One patient had partial loss of vision in one eye. Though all four patients were completely relieved of their myalgic pains by these drugs, their continued use, by masking progression of the disease, creates a hazard.

The Patients

Case 1

A woman, aged 57 years, with no history of earlier rheumatic disease, had headache and muscular pains in her calves for a short while in 1962. In January, 1963, the pain in her calves was so bad that she could not get out of her bed. She felt very tired and her temperature was slightly raised. The erythrocyte-sedimentation rate (E.S.R.) was 47 mm. in the first hour (Westergren); plasma-fibrinogen 0·8 g. per 100 ml. Oxyphenbutazone was given in a dose of 600 mg. a day for 2 days and then reduced to 300 mg. a day. During the week of treatment, the patient had no muscular pains; her temperature was still raised, however, and her E.S.R. was almost doubled to 91 mm. A temporal-artery biopsy specimen on microscopy was suggestive of arteritis. Prednisone was given instead of oxyphenbutazone, and within a few days the temperature and E.S.R. returned to normal.

Case 2

A man, aged 70 years, was admitted to another hospital in January, 1965, on account of bitemporal headache and fever; his E.S.R., was 100 mm. in the first hour. After 3 days on phenylbutazone 600 mg. a day his temperature was normal and he was completely relieved of his pains. 4 days after he left the hospital he was still free of pain when he noticed a defect of the visual field of his left eye. He returned to the hospital where now a bilateral thickening of his temporal arteries could be detected. Ophthalmoscopy revealed œdema of his left optic disc. The E.S.R. was 129 mm. The patient was sent to our clinic where biopsy of a temporal artery established the diagnosis of arteritis. The E.S.R. decreased rapidly with prednisolone therapy, but the visual-field defect was irreversible. In August, 1965, the treatment was interrupted by mistake; this was rapidly followed by a return of headache, fever, and raised E.S.R. The symptoms again rapidly subsided on reinstitution of steroid therapy.

Case 3

A woman, aged 68 years, was taken ill in August, 1965, with severe pains in the back of her neck, both knee-joints, and left shoulder. She also felt very tired and had lost 12 kg. of weight. The E.S.R. was 80 mm. in the first hour. Her temporal arteries were examined several times without any abnormal findings. All pain disappeared after treatment with oxyphenbutazone 300 mg. a day, subsequently reduced to 100–200 mg. a day without a return of the pain. In October—8 weeks after the therapy was started—we felt circumscribed thickening in relation to the branches of the temporal arteries. Microscopically, there was a typical giant-cell arteritis. The E.S.R. at the time of biopsy was 44 mm. The thickening disappeared with prednisone therapy, and the general condition of the patient improved rapidly.

Case 4

A woman, aged 75 years, was in hospital in July, 1965, on account of hæmatemesis, probably due to epistaxis. The E.S.R. was 80 mm. in the first hour; this induced a thorough search for malignancy without positive findings. Within a week of her leaving the hospital, she was almost totally immobilised by diffuse myalgic pains over all her extremities and trunk, including the back of her neck. The E.S.R. was 115 mm. Both temporal arteries were normal on palpation. Oxyphenbutazone was given in a dose of 600 mg. a day, reduced after a few days

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MIARECZKOWANIE WYŻSZYCH KWASÓW TŁUSZCZOWYCH W RÓŻNYCH ŚRODOWISKACH

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Jednym z najstarszych zastosowań środowiska niewodnego w analizie chemicznej jest oznaczenie zawartości kwasów tłuszczowych, polegające na ich alkacimetrycznym miareczkowaniu za pomocą wodorotlenku lub alkoholanu sodowego w chloroformie, czterochlorku węgla, benzenie i toluenie [1] a także w mieszaninach benzen-alkohol i innych [2]. Wybór środowiska niewodnego podyktowany jest koniecznością przeprowadzenia do roztworu substancji tłuszczowych, które są praktycznie nierozpuszczalne w wodzie. Jakkolwiek powyższa metoda oznaczania kwasów tłuszczowych, z niewielkimi tylko modyfikacjami, przetrwała do dnia dzisiejszego [3,4], to jednak wydaje się słuszne ponowne jej rozpatrzenie zgodnie z kryteriami nowoczesnej teorii analizy w środowisku niewodnym [5–7].

Literatura dotycząca miareczkowania słabych kwasów w rozpuszczalnikach niewodnych jest już dziś bardzo bogata; oddziaływanie środowiska na przebieg reakcji kwas—zasada i na kształt krzywej miareczkowania tłumaczy się w pierwszym rzędzie wpływem własności protonoakceptorowych rozpuszczalnika oraz wartością jego stałej dielektrycznej [6,7]. Parametry te tylko w sposób bardzo ogólny ujmują wspomnianą zależność; istotną rolę odgrywają również inne czynniki jak np. budowa strukturalna cząsteczki rozpuszczalnika [8], powstawanie podczas zobojętniania międzycząsteczkowych wiązań wodorowych [9] i wiele innych zjawisk [10].

Dobór odpowiedniego rozpuszczalnika nie jest więc prosty i łatwy do przewidzenia — w przypadku zaś miareczkowania wyższych kwasów tłuszczowych — komplikuje się dodatkowo z powodu słabej rozpuszczalności produktów reakcji, a więc możliwości wytrącania osadu podczas miareczkowania.

W niniejszej pracy przeprowadzono szereg miareczkowań w rozpuszczalnikach różniących się własnościami chemicznymi i wartością stałej dielektrycznej. Wyniki tych pomiarów posłużyły następnie do określenia wpływu tych właściwości na przebieg miareczkowania kwasów tłuszczowych i do oceny praktycznej przydatności badanych rozpuszczalników.

CZĘŚĆ DOŚWIADCZALNA

Aparatura

Pehametr lampowy LBS-66 i pehametr Tacussel-U8 sprzężony z rejestratorem.

Układ elektrod szklana-kalomelowa zmodyfikowana[11] (nasycony wodny roztwór KCl zastąpiono nasyconym roztworem KCl w metanolu).

Mieszadło magnetyczne z „bączkiem" zatopionym w szkło.

Biureta z podziałką co 0,05 ml.

Odczynniki

Pirydyna, metanol, benzen, aceton, izopropanol (POCh-Gliwice) — oczyszczone i odwodnione wg przepisu podanego w literaturze[12].

N.N.-Dwumetyloformamid (VEB Laborchemie Apolda).

Metanolan sodowy (Touzart et Matignon, Paris).

β-Etoksy-etanol (LOBA-Chemie, Wien).

Kwas palmitynowy, stearowy, linolowy, erukowy, olejowy (Touzart et Matignon Paris).

Kwas mirystynowy, (Laboratoires du Bois de Boulogne).

Kwas kaprylowy, pelargonowy (BDH Laboratory Reagent).

Metanolan sodowy, 0,1 N roztwór w mieszaninie metanol-benzen (1+4) (5,4 g metanolanu sodowego rozpuszczono w 200 ml metanolu i 800 ml benzenu; miano nastawiano na kwas benzoesowy w pirydynie).

Miareczkowanie potencjometryczne

Odważkę ok. 0,5 miligramcrównoważnika oznaczanej substancji rozpuszczono w 40 ml rozpuszczalnika i miareczkowano potencjometrycznie 0,1 N roztworem metanolanu sodowego z układem elektrod szklana—kalomelowa zmodyfikowana. Metanolan sodowy dodawano z biurety początkowo co 0,2—0,3 ml, a w okolicach punktu równoważnikowego co 0,05 ml, odczekując każdorazowo kilkanaście sekund przed odczytem wskazań miliwoltomierza. Przed pomiarem do roztworu dodawano kilka miligramów LiCl w celu szybszego uzyskania potencjału równowagowego elektrody wskaźnikowej. Przy pracy z pirydyną i dwumetyloformamidem roztwór ponadto barbotowano azotem aby nie dopuścić doń dwutlenku węgla. Skład roztworu w punkcie równoważnikowym przy zachowaniu opisanych wyżej warunków pomiaru, stanowił około 90% badanego rozpuszczalnika.

Punkt równoważnikowy miareczkowania wyznaczano metodą Hahna.

OMÓWIENIE WYNIKÓW

W tablicy 1 zestawiono wyniki pomiarów. Różnica między wielkością odważki, a znalezionym wynikiem (średnia z trzech pomiarów) daje pewien obraz dokładności metody. Ujemne na ogół wartości błędów wskazują na to, że stosowane próbki mogły zawierać pewną ilość zanieczyszczeń. Oceny przydatności rozpuszczalników dokonywano przede wszystkim w oparciu o kształt krzywych potencjometrycznych, szczególnie o ich przebieg w pobliżu punktu równoważnikowego. Na tej podstawie oszacowano precyzję pomiarów.

W każdym z rozpuszczalników zmiareczkowano 8 wyższych kwasów tłuszczowych. Kwasy te rozpuszczały się w stosowanych rozpuszczalnikach bardzo dobrze, natomiast produkty reakcji — mydła rozpuszczały się tylko w alkoholu β-etoksyetylowym. Mydła te niekiedy w postaci koloidalnego żelu zaczynały się wytrącać pod koniec miareczkowania poważnie opóźniając ustalenie się potencjału. Miało to prawdopodobnie wpływ na zmniejszenie się w niektórych rozpuszczalnikach skoku po-

Tablica 1. Wy
z 3 p

Lp.	
1	Ste
2	Lin
3	Ole
4	Pa
5	Pe
6	K

Tablica 1. Wyniki miareczkowania kwasów tłuszczowych w różnych rozpuszczalnikach (średnie z 3 pomiarów)

Lp.	Kwas	Rozpuszczalnik	Wzięto g	Znaleziono g	Błąd %
1	Stearowy	izopropanol	0,1532	0,1509	−1,50
		metanol-benzen	0,1608	0,1592	−0,99
		aceton	0,1427	0,1402	−1,75
		β-etoksyetanol	0,1419	0,1429	+0,70
		pirydyna	0,1269	0,1278	+0,71
		dwumetyloformamid	0,1532	0,1508	−1,56
2	Linolowy	izopropanol	0,1287	0,1283	−0,31
		metanol-benzen	0,1324	0,1337	+0,98
		aceton	0,1366	0,1345	−1,54
		β-etoksyetanol	0,1200	0,1208	+0,67
		pirydyna	0,1345	0,1330	−1,10
		dwumetyloformamid	0,1229	0,1219	−0,81
3	Olejowy	izopropanol	0,1332	0,1316	−1,21
		metanol-benzen	0,1324	0,1337	+0,98
		aceton	0,1470	0,1452	−1,22
		β-etoksyetanol	0,1331	0,1324	−0,52
		pirydyna	0,1297	0,1282	−1,16
		dwumetyloformamid	0,1310	0,1325	+1,01
4	Palmitynowy	izopropanol	0,1295	0,1287	−0,62
		metanol-benzen	0,1188	0,1179	−0,71
		aceton	0,1104	0,1128	+2,18
		β-etoksyetanol	0,1144	0,1128	−1,22
		pirydyna	0,1265	0,1248	−1,34
		dwumetyloformamid	0,1288	0,1283	−0,39
5	Pelargonowy	izopropanol	0,0856	0,0841	−1,75
		metanol-benzen	0,8884	0,8865	−2,14
		aceton	0,0638	0,0652	+2,20
		β-etoksyetanol	0,0721	0,0713	−1,11
		pirydyna	0,0795	0,0782	−1,64
		dwumetyloformamid	0,0847	0,0832	−1,77
6	Kaprylowy	izopropanol	0,0658	0,0653	−0,73
		metanol-benzen	0,0642	0,0649	+1,09
		aceton	0,0722	0,0718	−0,55
		β-etoksyetanol	0,0933	0,0930	−0,32
		pirydyna	0,0627	0,0622	−0,80
		dwumetyloformamid	0,0674	0,0678	+0,59

dalszy ciąg tabl. 1

Lp.	Kwas	Rozpuszczalnik	Wzięto g	Znaleziono G	Błąd %
7	Mirystynowy	izopropanol	0,0972	0,0962	−1,03
		metanol-benzen	0,0984	0,0989	+0,51
		aceton	0,1141	0,1153	+1,05
		β-etoksyetanol	0,1109	0,1121	+1,08
		pirydyna	0,1087	0,1067	−1,84
		dwumetyloformamid	0,0965	0,0942	−1,34
8	Erukowy	izopropanol	0,1783	0,1768	−0,84
		metanol-benzen	0,1898	0,1875	−1,21
		aceton	0,1672	0,1648	−1,43
		β-etoksyetanol	0,1756	0,1748	−1,02
		pirydyna	0,1509	0,1500	−0,60
		dwumetyloformamid	0,1718	0,1698	−1,16

tencjału w punkcie równoważnikowym, który to skok na ogół nie przekraczał 250 mV. Najsłabiej rozpuszczalne okazały się sole sodowe kwasów stearowego i palmitynowego wytrącające się już po dodaniu pierwszych porcji metanolanu sodowego, zawiesina ich jednak w przeciwieństwie do soli kwasów nienasyconych lub o niższym ciężarze cząsteczkowym miała postać zbitą i prawie krystaliczną. Niewątpliwie wpłynęło to w połączeniu z nieco silniejszym charakterem kwasowym kwasów stearowego i palmitynowego na najwyższy skok potencjału we wszystkich użytych rozpuszczalnikach.

Na przykładzie kwasów o długości łańcucha węglowego C_{18}, różniących się od siebie tylko stopniem nienasyconości przedstawiono (rys. 1, 2 i 3) krzywe miareczkowania w pobliżu punktu równoważnikowego. Pozostały przebieg krzywych nie różnił się w zasadzie od normalnego przebiegu, charakterystycznego dla miareczkowania słabych kwasów mocną zasadą.

I tak na przykład w przypadku miareczkowania kwasów tłuszczowych w acetonie po punkcie równoważnikowym następował spadek potencjału, dając maksimum na odpowiednich krzywych, być może w wyniku rozkładu acetonu w środowisku silnie zasadowym [5].

W przypadku miareczkowania kwasów linolowego i olejowego w pirydynie przy pierwszych porcjach dodanego metanolanu sodowego następował dość silny wzrost potencjału, który następnie malał przed skokiem w punkcie równoważnikowym. Stąd nieco różny od innych (schodkowy) obraz krzywych w tym rozpuszczalniku.

Zdecydowanie największy skok potencjału — i to dla wszystkich kwasów tłuszczowych — występuje w izopropanolu i nieco mniejszy zaś w acetonie; rozpuszczalniki te stanowią więc odpowiedniejsze środowisko do miareczkowania kwasów tłuszczowych aniżeli stosowana częściej mieszanina metanol—benzen, gdzie skok potencjału jest niższy, a przebieg krzywej w tym obszarze mniej stromy. Stosunkowo dobrym rozpuszczalnikiem okazała się pirydyna. Skok krzywej potencjometrycznej był co prawda niższy niż w izopropanolu, ale za to bardzo wyraźny i pozwalający

Rys. 1. Krzyw
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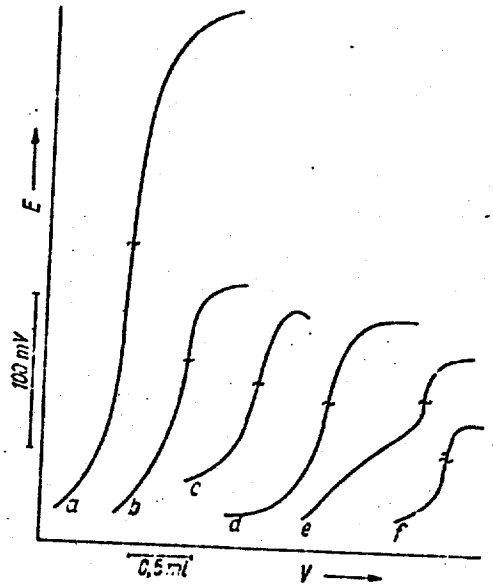
kano w ten spo
stałej dielektry
pozwalają na
wszystkich por

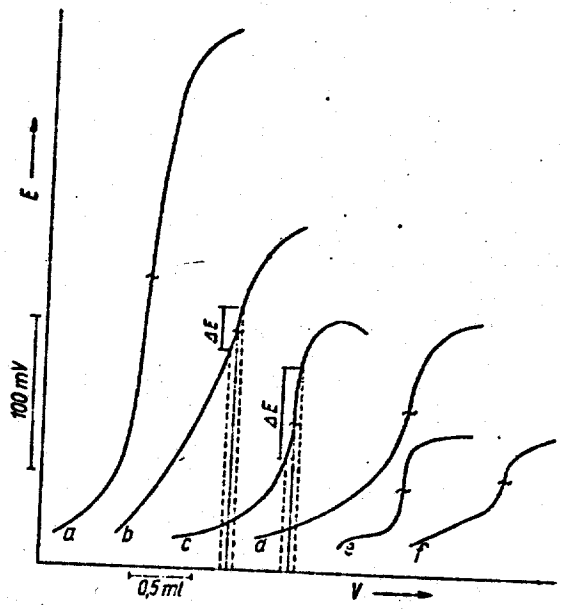
Rys. 1. Krzywe potencjometryczne miareczkowania kwasu stearowego 0,1 N metanolanem sodowym w różnych rozpuszczalnikach; *a* — izopropanol; *b* — β-etoksyetanol; *c* — aceton; *d* — metanol-benzen (1÷4); *e* — pirydyna; *f* — N. N.-dwumetyloformamid

na dokładne wyznaczenie punktu równoważnikowego. Znane są jednak wady tego rozpuszczalnika w metodzie miareczkowania alkacymetrycznego — duża higroskopijność i szybkie pochłanianie dwutlenku węgla.

Na uwagę zasługuje także alkohol β-etoksyetylowy nie tylko ze względu na dość wysoki skok potencjału w punkcie równoważnikowym, ale i na lepszą rozpuszczalność produktu zobojętnienia. Zdecydowanie najniższy i najmniej wyraźny skok stwierdzono w dwumetyloformamidzie, co jest pewnym zaskoczeniem zważywszy na jego zasadowość i dość wysoką stałą dielektryczną, które stwarzają wg przewidywań dobre warunki dla miareczkowania słabych kwasów. Przyrost skoku potencjału w punkcie równoważnikowym ΔE został umownie zmierzony w granicach $\pm$ 1,0% objętości odczynnika miareczkującego tj. w granicach 99,0—101,0% ilości stechiometrycznej. Sposób wyznaczania ΔE widoczny jest na rys. 3. Uzyskane w ten sposób wartości ΔE zestawiono w tablicy 2 wraz z wartościami stałej dielektrycznej rozpuszczalników i ich zasadowością. Wartości ΔE pozwalają na oszacowanie precyzji pomiarów. Są one najwyższe dla wszystkich pomiarów wykonanych w izopropanolu.



Rys. 2. Krzywe potencjometryczne miareczkowania kwasu olejowego 0,1 N metanolanem sodowym w różnych rozpuszczalnikach. Oznaczenie jak na rys. 1



Rys. 3. Krzywe potencjometryczne miareczkowania kwasu linolowego 0,1 N metanolanem sodowym w różnych rozpuszczalnikach. Oznaczenie jak na rys. 1.

Opisane wyniki doświadczeń wskazują, że wpływ wartości stałej dielektrycznej i własności protonoakceptorowych rozpuszczalnika na przebieg miareczkowania alkacymetrycznego kwasów tłuszczowych nie jest tak duży jak można się było tego spodziewać. Świadczy o tym m.in. zaobserwowany w izopropanolu najwyższy skok potencjału, mimo że roz-

Tablica 2. Przyrosty potencjału ΔE na $\pm$ 1% całkowitej objętości rozpuszczalnika miareczkującego w punkcie równoważnikowym

Rozpuszczalnik	Przyrost potencjału ΔE mV			Stała dielektryczna rozpuszczalnika	Zasadowość rozpuszczalnika
	kwas stearowy	kwas linolowy	kwas olejowy		
Pirydyna	15	20	12	12,3	
N.N.-Dwumetyloformamid	20	10	10	36,7	wzrost własności zasadowych
Izopropanol	80	45	40	18,3	
β-etoksyetanol	45	25	10	—	
Aceton	20	10	18	20,7	
Benzen				2,3	
Metanol	22	15	15	32,6	

puszczalnik ten ma stosunkowo niewielką stałą dielektryczną i nie odznacza się dużą zasadowością.

Duży wpływ na przebieg krzywych potencjometrycznych ma powstawanie osadu podczas miareczkowania. Tym tłumaczy się między innymi mało wyraźny skok potencjału w przypadku dwumetyloformamidu, spowodowany prawdopodobnie gorszą pracą układu elektrod w tym rozpuszczalniku, gdzie powstający podczas dodawania metanolanu sodowego osad miał charakter galaretowaty. Najlepszym spośród zbadanych rozpuszczalników okazał się izopropanol. Odczynnik ten jest stosunkowo dobrym rozpuszczalnikiem tłuszczów jest ponadto bezpieczny w pracy, dostępny w handlu i z tych względów godzien jest polecenia.

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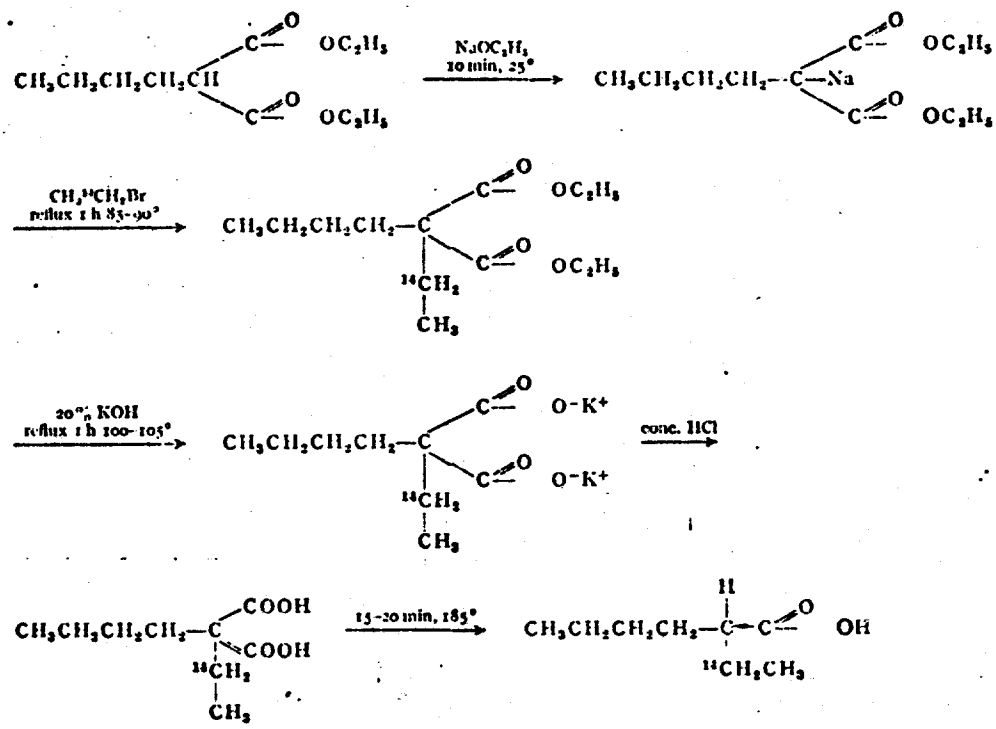

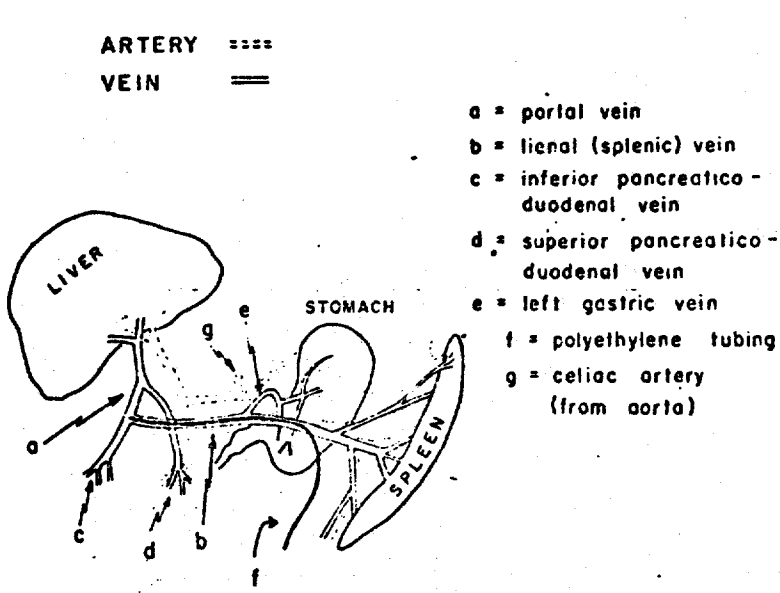

Fig. 1. Diagram showing the position of the portal vein fistula.

(splenic) vessels (vein as well as artery) were ligated about one-quarter of an inch from the point of junction of the lienal vein with the left gastric vein, and a second loose ligature was placed below the first. Polyethylene tubing (PE 10), about 8 inches long, was inserted into the lienal vein so that the tip of the tubing extended into the

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TITRATION OF HIGHER FATTY ACIDS IN VARIOUS MEDIA

Maria ŁUGOWSKA and Jarosław FLIGIER

Department of Technical Analysis, Politechnika, Gliwice

Eight of higher fatty acids were potentiometrically titrated with sodium methanolate solution using glass and modified calomel electrodes.

Isopropanol, acetone, methanol-benzene mixture (1+4), β-ethoxyethanol, pyridine, and N,N-dimethylformamide were used as solvents. Potentiometric titration curves were given and the effect of chemical and dielectric properties of the solvent on their shape was discussed. It was pointed out that formation of insoluble precipitate in the course of reaction is undesirable. Potential step at equivalence point in isopropanol and acetone was greater than that obtained in methanol-benzene mixture which was most often used hitherto for the titration of higher fatty acids.

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J. Clin. Invest. 37 (10): 1379-93 (1958)

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STUDIES ON KETONE METABOLISM IN MAN. II. THE EFFECT OF GLUCOSE, INSULIN, CORTISONE AND HYPOGLYCEMIA ON SPLANCHNIC KETONE PRODUCTION [1]

By HARRY T. McPHERSON,[2] EMILE E. WERK, JR.,[3, 4] JACK D. MYERS,[5] AND FRANK L. ENGEL

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(Submitted for publication April 14, 1958; accepted June 19, 1958)

We have previously described a technique which utilizes hepatic venous catheterization for the estimation of net splanchnic ketone body production (NSKP) in man (1). In a series of subjects without metabolic abnormality, a small but significant endogenous NSKP was detected in the postabsorptive state, and upon the intravenous administration of a ketone body precursor, sodium octanoate, the NSKP was increased fivefold. The present report concerns the application of this technique to the study of the effects of glucose, insulin, cortisone and hypoglycemia on ketone body production in man.

METHODS

Twenty-seven mildly ill male hospital patients between the ages of 23 and 58 years, without metabolic abnormality, were used as subjects and studied after a 12 to 14 hour fast. The criteria of selection and management of subjects, technique of hepatic venous catheterization, methods of chemical analysis, and calculations are identical to those previously reported (1). The 16 normal males in the study will serve as the control series, designated "1955 series," for the present experiments.

The subjects of the present experiments were divided into four groups and studied in a manner similar to the "1955 series" except for the specific metabolic alterations noted below for each group under "Results." The general plan of study for all groups was as follows: After mild phenobarbital sedation, inlying femoral arterial and forearm venous needles and the hepatic venous catheter were inserted. An intravenous infusion of bromsulphalein was started and maintained throughout the period of study. A control study was made on each subject during which femoral arterial and hepatic venous bloods were each sampled four times at five minute intervals for the subsequent determination of bromsulphalein, glucose and total ketone concentrations. A 500 ml. infusion of a 1.5 per cent solution of sodium octanoate was then administered intravenously at a constant rate over a one hour period, during which time blood sampling was repeated four times at 15 minute intervals. Blood sampling for oxygen content and saturation was interspersed at appropriate intervals during each period. The estimated hepatic blood flow (EHBF) was determined by the technique of Bradley, Ingelfinger, Bradley and Curry (2). The net splanchnic glucose production (NSGP) and net splanchnic ketone production (NSKP) are the products of the hepatic blood flow and the hepatic venous-arterial blood glucose and ketone differences, respectively. The net splanchnic oxygen consumption (SPO_2) was calculated by multiplying the arterial-hepatic venous blood oxygen differences by the EHBF.

In addition to the above studies, peripheral venous blood total ketones and glucose concentrations during a four hour insulin tolerance test (0.1 unit per Kg., I.V.) were estimated in 11 normal medical students with and without 200 mg. of cortisone acetate being administered orally one hour prior to the insulin.

RESULTS

1955 series

The data, including the mean plus or minus standard error and standard deviation, for the control series of 16 male subjects previously reported in detail (1), are given at the bottom of Table I. Each subject was studied first in the postabsorptive state, the *control period*, and then during the hour-long infusion of 500 ml. of 1.5 per cent sodium octanoate solution, henceforth designated the *octanoate period*.

Glucose series (Table I)

Six subjects were studied in a manner differing from the 1955 series only in that 20 Gm. of glucose was mixed with the octanoate infusion. Thus, as expected, the data of the control period were entirely comparable to those in the 1955 series of

[1] Supported in part by research grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service (A 142), and from Merck and Company, Rahway, N. J.

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TABLE I

The metabolic effects of intravenously administered sodium octanoate mixed with glucose

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.-F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.-F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.-F.A.
yrs. H. M. 37	Functional G.I. 1.71	1+	22.5	30.3	7.8	58.0	80.0	22.0	+35.5	+49.7	+14.2
W. H. 28	Duodenal ulcer 1.59	1+	11.0	15.8	4.8	45.2	48.2	3.0	+34.2	+32.4	− 1.8
J. F. 31	Duodenal ulcer 1.66	1+	14.5	19.2	4.7	52.5	65.2	12.8	+38.0	+46.0	+ 8.1
H. K. 52	Duodenal ulcer 1.91	1+	13.8	15.8	2.0	34.5	51.5	17.0	+20.7	+35.7	+15.0
T. G. 28	Cervical disc 1.77	1+	9.5	11.8	2.3	27.3	36.8	9.5	+17.8	+25.0	+ 7.2
B. U. 30	Functional G.I. 1.98	1+	10.0	16.0	6.0	25.8	41.5	15.7	+15.8	+25.5	+ 9.7
Mean age, 34 Range, 28-52	Mean S.A., 1.77	Mean S.E. S.D.	13.6 1.97 4.83	18.1 2.81 6.89	4.5 0.87 2.14	40.6 5.46 13.39	53.9 6.57 16.09	13.3 2.68 6.57	+27.0 4.05 9.93	+35.8 4.22 10.34	+ 8.8 2.45 6.00
Statistical comparison with control series		p	>0.5	>0.5	>0.5	>0.5	>0.05 <0.1	<0.01	>0.5	>0.05 <0.1	<0.01
Previously reported control series (16 male subjects)		Mean S.E. S.D.	14.1 1.73 6.92	18.4 2.24 8.96	4.3 0.62 2.46	42.6 2.56 10.25	64.8 2.94 11.79	22.2 1.10 4.40	+28.5 2.26 9.01	+46.4 2.83 11.31	+17.9 1.19 4.77

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs. H. M. 37	Functional G.I. 1.71	1+	808	828	+ 20	62	182	+120	34	39	+ 5
W. H. 28	Duodenal ulcer 1.59	1+	548	556	+ 8	28	17	− 11	33	40	+ 7
J. F. 31	Duodenal ulcer 1.66	1+	740	751	+ 11	35	96	+ 61	29	32	+ 3
H. K. 52	Duodenal ulcer 1.91	1+	694	548	−146	16	88	+ 72	28	29	+ 1

TABLE I—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
T. G. 28	Cervical disc 1.77	1+	1,106	1,238	+132	28	126	+ 98	36	42	+ 6
B. U. 30	Functional G.I. 1.98	1+	764	819	+ 55	46	129	+ 83	35	46	+11
Mean age, 34	Mean	Mean	777	790	+ 13	35.8	106.3	70.5	32.5	38	+ 5.5
	S.A.,	S.E.	75.3	102.9	31.6	6.60	22.4	18.34	1.35	2.59	1.41
Range, 28–52	1.77	S.D.	184.0	252.1	84.8	16.2	54.8	44.9	3.31	6.36	3.46
Statistical comparison with control series		p	>0.5	>0.3 <0.4	>0.1 <0.2	>0.5	<0.01	>0.01 <0.02	>0.5	>0.4 <0.5	>0.5
Previously reported control series (16 male subjects)		Mean	790	732	− 58.0	34.0	158.0	+124	36.1	40.3	+ 4.2
		S.E.	38.0	40.1	27.15	5.20	6.86	7.36	2.05	1.52	1.46
		S.D.	151.7	160.4	108.6	20.86	27.46	41.4	7.94	5.83	5.66

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
H. M. 37	Functional G.I. 1.71	1+	14	89	149	+60	50	−30	− 80	94	94	0
W. H. 28	Duodenal ulcer 1.59	1+	0	81	149	+68	62	− 8	− 70	85	83	−2
J. F. 31	Duodenal ulcer 1.66	1+	7	89	140	+51	65	−10	− 75	90	84	−6
H. K. 52	Duodenal ulcer 1.91	1+	10	87	131	+44	72	4	− 68	99	93	−6
T. G. 28	Cervical disc 1.77	1+	12	94	160	+66	96	−41	−137	86	78	−8
B. U. 30	Functional G.I. 1.98	1+	11	81	128	+47	64	−10	− 74	100	103	+3
Mean age, 34	Mean	Mean	9.0	86.6	143	56.4	68.2	−15.8	− 84	92.3	89.2	−3.1
	S.A.,	S.E.	2.02	2.04	4.96	4.12	6.28	6.72	10.73	2.61	3.74	1.73
Range, 28–52	1.77	S.D.	4.96	5.00	12.15	10.1	15.40	16.50	26.30	6.40	9.16	4.23
Statistical comparison with control series		p	<0.01	>0.5	<0.01	<0.01	>0.5	<0.01	<0.01	>0.1 <0.2	>0.2 <0.3	>0.5
Previously reported control series (16 male subjects)		Mean	14.9	84.6	81.6	− 3.0	68.0	65.0	− 3.0	84.4	82.2	−3.4
		S.E.	0.88	1.87	1.76	0.5	4.47	6.0	5.48	3.19	3.32	0.73
		S.D.	3.46	7.46	7.05	2.01	17.92	24.08	21.82	12.81	13.04	2.85

TABLE II

The metabolic effect of intravenously administered sodium octanoate mixed with glucose and insulin

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.–F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.–F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs.											
C. M. 39	Cluster headache 1.72	2+	11.0	11.2	0.2	26.7	37.3	10.6	+15.7	+26.1	+10.4
A. H. 28	Duodenal ulcer 1.92	2+	13.5	17.8	4.3	33.0	44.0	11.0	+19.5	+26.2	+ 6.7
A. W. 41	Low back pain 1.76	1+	9.5	12.0	2.5	30.0	48.0	18.0	+20.5	+36.0	+15.5
P. S. 47	Peptic ulcer 1.78	2+	11.0	17.2	6.2	34.0	58.2	24.2	+23.0	+41.0	+18.0
G. F. 33	Epilepsy 1.84	1+	4.2	6.5	2.3	22.0	35.0	13.0	+17.8	+28.5	+10.7
J. G. 30	Peptic ulcer 1.87	1+	12.0	14.7	2.7	31.8	48.5	16.7	+19.8	+33.8	+14.0
C. L. 23	Arrested coccidio-mycosis 1.92	1+	8.8	14.0	5.2	24.5	38.2	13.7	+15.7	+24.2	+ 8.5
E. E. 31	Duodenal ulcer 1.75	1+	36.2	50.8	14.6	52.0	79.5	27.5	+15.8	+28.7	+12.9
Mean age, 34	Mean S.A., 1.82	Mean	13.3	18.0	4.7	31.8	48.6	16.8	+18.5	+30.6	+12.1
		S.E.	3.41	4.85	1.54	3.25	5.15	2.18	1.04	1.98	1.31
Range, 23–47		S.D.	9.66	13.72	4.37	9.20	14.58	6.16	2.94	5.61	3.72
Statistical comparison with control series			>0.5	>0.5	>0.5	>0.02 <0.05	<0.01	>0.02 <0.05	<0.01	<0.01	<0.01

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
C. M. 39	Cluster headache 1.72	2+	731	766	+ 35	3	81	+ 78	34	42	+ 8
A. H. 28	Duodenal ulcer 1.92	2+	1,088	1,147	+ 59	46	132	+ 86	31	38	+ 7
A. W. 41	Low back pain 1.76	1+	623	574	− 49	16	100	+ 84	33	33	0
P. S. 47	Peptic ulcer 1.78	2+	820	689	−131	52	167	+115	33	34	+ 1

TABLE II—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic-ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
G. F. 33	Epilepsy 1.84	1+	627	632	+ 5	14	82	+ 68	28	38	+10
J. G. 30	Peptic ulcer 1.87	1+	631	633	+ 2	18	106	+ 88	25	34	+ 9
C. L. 23	Arrested coccidio-mycosis 1.92	1+	678	775	+ 97	34	108	+ 74	38	43	+ 5
E. E. 31	Duodenal ulcer 1.75	1+	763	815	+ 52	110	223	+113	37	45	+ 8
Mean age, 34	Mean S.A., 1.82	Mean	745	754	+ 9	36.6	125.0	+ 88.4	32.4	38.3	+ 5.9
		S.E.	54.8	63.25	23.70	12.06	17.14	6.07	1.54	1.59	1.31
Range, 23–47		S.D.	155.0	179.0	67.09	34.12	48.52	17.19	4.36	4.49	3.73
Statistical comparison with control series			>0.5	>0.5	>0.1 <0.2	>0.5	>0.02 <0.05	<0.01	>0.2 <0.3	>0.3 <0.4	>0.4 <0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones %	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.												
C. M. 39	Cluster headache 1.72	2+	9	80	106	+26	55	− 7	− 62	79	72	−7
A. H. 28	Duodenal ulcer 1.92	2+	11	86	113	+27	73	+13	− 60	96	91	−5
A. W. 41	Low back pain 1.76	1+	10	82	93	+11	−1	− 2	− 1	70	61	−9
P. S. 47	Peptic ulcer 1.78	2+	14	80	102	+22	58	+ 4	− 54	91	90	−1
G. F. 33	Epilepsy 1.84	1+	8	82	94	+12	91	16	− 75	84	84	0
J. G. 30	Peptic ulcer 1.87	1+	11	82	111	+29	136	+ 2	−134	74	65	−9
C. L. 23	Arrested coccidio-mycosis 1.92	1+	9	79	111	+32	55	0	− 55	79	79	0
E. E. 31	Duodenal ulcer 1.75	1+	13	77	122	+45	56	0	− 56	86	85	−1
Mean age, 34	Mean S.A., 1.82	Mean	10.7	80.9	106.5	+25.6	65.4	3.4	− 62.0	82.4	78.4	−4.0
		S.E.	0.66	0.99	3.49	3.87	13.66	2.67	12.86	3.30	3.99	1.40
Range, 23–47		S.D.	1.88	2.80	9.89	10.95	38.66	7.56	36.38	9.35	11.29	3.96
Statistical comparison with control series			<0.01	>0.1 <0.2	<0.01	<0.01	>0.5	<0.01	<0.01	>0.5	>0.5	>0.5

TABLE III

Total blood ketone and sugar levels during insulin tolerance tests (0.1 unit insulin per Kg. body weight) in 11 normal medical students with and without cortisone pretreatment

		Total blood ketones μM acetone/100 ml. blood						Blood sugar mg./100 ml. blood					
		Fasting	Δ ½ hr.	Δ 1 hr.	Δ 2 hrs.	Δ 3 hrs.	Δ 4 hrs.	Fasting	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs
Control study	Mean	9.3	−2.4	−2.4	+3.8	+12.9	+16.2	88.0	35.6	67.1	85.4	86.6	85.6
	S.E.	1.38	1.20	1.20	1.72	2.75	3.44	1.66	2.76	3.90	1.90	1.61	1.61
	S.D.	4.47	3.96	3.96	5.50	8.94	11.70	5.51	9.16	12.95	6.30	5.34	5.34
Statistical comparison with fasting levels	p		>0.05 <0.1	>0.05 <0.1	>0.05 <0.1	<0.01	<0.01						
After cortisone	Mean	8.0	−2.4	−0.9	+1.0	+10.7	+12.9	88.7	33.1	70.1	90.4	99.2	99.4
	S.E.	1.38	0.86	1.38	0.86	2.41	3.27	1.46	2.20	4.49	2.44	1.72	1.96
	S.D.	4.30	2.92	4.30	2.75	8.08	11.01	4.84	7.31	14.91	8.12	5.72	6.50
Statistical comparison with fasting level	p		>0.01 <0.02	>0.5	>0.2 <0.3	<0.01	<0.01						
Statistical comparison with control study (p)		>0.5	>0.5	>0.3 <0.4	>0.1 <0.2	>0.5	>0.5	>0.5	>0.4 <0.5	>0.5	>0.1 <0.2	<0.01	<0.01

subjects. After the glucose-octanoate infusion, there was the anticipated elevation of the mean femoral arterial blood glucose level to 143 ± 4.96 (S.E.) mg. per cent and a complete suppression of net splanchnic glucose production. The data on ketone body metabolism in the octanoate period differed from those in the corresponding period in the 1955 series in the following ways: *1*) There was a significant decrease ($p < 0.01$) in the hepatic venous-femoral arterial (HV-FA) blood ketone difference, which was 13.3 ± 2.68 μM per cent compared to 22.2 ± 1.10 μM per cent in the 1955 series; *2*) the mean NSKP was significantly lower ($p < 0.01$) than in the 1955 series, being 106.3 ± 22.4 and 158.0 ± 6.86 μM per minute per $M.^2$, respectively; and *3*) the per cent of infused octanoate estimated to be converted to ketone bodies was 9.0 ± 2.02 per cent, a value significantly less ($p < 0.01$) than the 14.9 ± 0.88 per cent found in the 1955 series. In one subject, H.M., the HV–FA blood ketone difference, NSKP and per cent conversion were not significantly different from the 1955 series. In all other subjects the differences were distinct. There were no significant differences in EHBF, SPO_2 or BSP clearance.

Glucose-insulin series (*Table II*)

Eight subjects were studied before and after the infusion of 20 Gm. of glucose plus 0.1 unit per Kg. body weight HGF-free insulin mixed with sodium octanoate, bringing out only minor differences from the glucose series. Again, the changes were limited to the octanoate infusion period. During the latter, arterial blood glucose levels were lower than those in the glucose series, but NSGP was equally effectively suppressed. Compared to the 1955 series, there were significantly smaller increases in mean arterial and hepatic-venous blood ketone levels and HV–FA blood ketone differences after octanoate infusion in this group ($p < 0.05$, < 0.01 and < 0.05). Similarly, the octanoate infusion induced a smaller net rise in NSKP than in the 1955 series, mean Δ NSKP being + 88.4 ± 6.07 and + 124 ± 7.36 μM per minute per $M.^2$, respectively ($p < 0.01$), and the estimated per cent conversion of octanoate to ketone bodies was reduced from 14.9 ± 0.88 per cent to 10.7 ± 0.66 per cent ($p < 0.01$). Although there was no significant difference in the octanoate-period NSKP between this and the glucose series, arterial blood ketone levels were lower in the insulin-glucose group ($p < 0.05$). Indirectly this might suggest that insulin promotes the pe-

ripheral utilization of ketone bodies in the presence of a large carbohydrate load, a concept in distinct contrast to the older observations of Chaikoff and Soskin (3) and Mirsky and Broh-Kahn (4) that neither glucose nor insulin affects the utilization of injected ketone bodies in the eviscerate animal.

While the mean NSKP in this group was significantly lower than that of the 1955 series in the octanoate period, two subjects, P.S. and E.E., attained a net rise in NSKP after octanoate infusion that was comparable to that seen in the 1955 series. In Subject E.E., however, the basal NSKP was approximately three times greater than that in the 1955 subjects. This finding, in the absence of starvation or alkalosis, might reflect the presence of greater anxiety, a potential ketogenic stimulus, than was recognized clinically. In Subject P.S., no explanation is apparent from the data.

Insulin tolerance tests in normal medical students (Table III)

Prior to the hepatic venous catheterization studies reported below in the "Hypoglycemic Series," a pilot study of blood sugar and ketone levels during a standard insulin tolerance test was carried out in 11 normal medical students after an overnight fast.

On the control day, marked hypoglycemia was attained in each subject, with a mean blood sugar level of 35.6 ± 2.76 mg. per cent one-half hour after the insulin. The total peripheral venous blood ketone concentrations showed no significant change from the fasting levels until three and four hours after insulin when there was a net rise of 12.9 ± 2.75 and 16.2 ± 3.44 μM per cent, respectively ($p < 0.01$ for both).[6]

Three days later, the tests were repeated one hour after 200 mg. of cortisone acetate had been administered orally. The degree of hypoglycemia at one-half hour and the rise in venous blood ketone levels during the third and fourth hours were not significantly different from the control day. Blood sugar values three and four hours after insulin (four to five hours after cortisone) rose to significantly higher levels ($p < 0.01$) than did those on the control day. On both the control and cortisone treatment days, there was a tendency for blood ketone concentrations to fall one-half hour after insulin, but this was statistically significant only on the cortisone day ($p < 0.02$). Cortisone pretreatment, in this dosage and timing, did not inhibit the rise in blood ketones during the third and fourth hours after insulin hypoglycemia.

Posthypoglycemic series (Table IV, Figure 1)

Brisk but brief insulin hypoglycemia was induced in six subjects at 7 a.m. by the rapid intravenous injection of 0.1 unit HGF-free insulin per Kg. body weight. Three hours later, at 10 a.m., each was studied in the same manner as the 1955 series before and during the infusion of sodium octanoate. Definite hypoglycemia, approximately 30 minutes in duration, was established in all subjects, the mean lowest blood sugar for the group being 31.0 ± 2.46 (S.E.) mg. per cent.

At the time of study by hepatic venous catheterization, the femoral arterial blood glucose levels and the NSGP had returned to normal control levels, but there were striking changes in ketone metabolism. There were significant increases in the arterial and hepatic venous blood ketone levels and a widening of the HV–FA blood ketone differences, the mean values being 38.8 ± 6.2, 57.6 ± 10.70 and 18.8 ± 5.30 μM per cent, compared to 14.1 ± 1.73, 18.4 ± 2.24 and 4.3 ± 0.62 μM per cent, respectively, in the 1955 series ($p < 0.01$, < 0.01 and < 0.05). Mean control period NSKP was 4.7 times greater than that in the 1955 series of subjects ($p < 0.01$). This is shown graphically in Figure 1. It is interesting to note that mean basal NSKP in the posthypoglycemic subject, 160.7 ± 46 μM per minute per $M.^2$, is almost identical with the mean octanoate period NSKP in the 1955 subjects, 158.0 ± 6.86 μM per minute per $M.^2$. If one could assume that the majority of new ketone bodies produced in the posthypoglycemic state arose from endogenous fatty acids, this might yield a rough estimation of the equivalent quantity of fatty acids being converted to ketones in this state. Despite the elevated basal ketone body levels, this group demonstrated approximately the same increase in blood ketone values

[6] In another group of medical students receiving norepinephrine I.V., there was a rise in total blood ketones of [illegible] per 100 ml. at the end of three hours. This rise was significantly less than that of the hypoglycemic groups ($p < 0.01$).

TABLE IV

Metabolic studies three hours after insulin hypoglycemia

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: Δ	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: Δ	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs. W. C. 42	Duodenal ulcer 1.77	2+	46.0	60.0	14.0	87.5	125.8	38.3[1]	+41.5	+65.8	+24.3
L. G. 58	Duodenal ulcer 1.71	2+	32.0	44.8	12.8	69.0	88.5	19.5	+37.0	+43.7	+ 6.7
G. K. 34	Duodenal ulcer 1.91	1+	45.5	71.5	26.0	80.8	112.5	31.7	+35.3	+41.0	+ 5.7
O. R. 30	Acne vulgaris 1.68	2+	51.8	92.8	41.0	76.5	128.5	52.0	+24.7	+35.7	+11.0
A. B. 33	Functional G.I. 1.99	1+	46.0	61.8	15.8	67.8	98.2	30.4	+21.8	+36.4	+14.6
H. H. 33	Asthma 1.49	1+	11.2	14.8	3.6	42.0	71.0	29.0	+30.8	+56.2	+25.4
Mean age, 38 Range, 30–58	Mean S.A., 1.76	Mean S.E. S.D.	38.8 6.12 15.0	57.6 10.70 26.30	18.8 5.30 13.0	70.6 6.40 15.82	104.1 9.1 22.4	33.5 4.4 10.9	+31.8 3.08 7.55	+46.5 4.9 12.0	+14.7 3.49 8.49
Statistical comparison with control series		p	<0.01	<0.01	>0.02 <0.05	<0.01	<0.01	<0.01	>0.4 <0.5	>0.5	>0.1 <0.2

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.[2] Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.[2] Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.[2] Control	During sod. oct.	Δ
yrs. W. C. 42	Duodenal ulcer 1.77	2+	903	866	−37	126	330	+204	30	32	+2
L. G. 58	Duodenal ulcer 1.71	2+	868	790	−78	111	154	+ 43	36	44	+8
G. K. 34	Duodenal ulcer 1.91	1+	914	826	−88	238	261	+ 23	44	37	−7
O. R. 30	Acne vulgaris 1.68	2+	836	825	−11	345	441	+ 96	43	43	0
A. B. 33	Functional G.I. 1.99	1+	749	801	+52	118	230	+112	36	42	+6
H. H. 33	Asthma 1.49	1+	716	644	−72	26	182	+156	27	28	+1

TABLE IV—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
Mean age, 38	Mean	Mean	831	792	−39	160.7	266.3	+105.6	36	37.7	+1.70
	S.A.,	S.E.	33.4	31.5	21.63	46.0	43.5	27.8	2.70	2.65	2.12
Range, 30–58	1.76	S.D.	81.8	77.1	53.0	112.8	106.5	68.0	6.60	6.50	5.20
Statistical comparison with control series		p	>0.1 <0.2	>0.1 <0.2	>0.5	<0.01	<0.01	>0.5	>0.5	>0.3 <0.4	>0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
W. C. 42	Duodenal ulcer 1.77	2+	24	82	74	−8	61	43	−18	80	81	+1
	Duodenal ulcer 1.71	2+	5	85	80	−5	67	47	−20	97	97	0
G. K. 34	Duodenal ulcer 1.91	1+	3	97	91	−6	64	55	− 9	108	108	0
O. R. 30	Acne vulgaris 1.68	2+	11	94	88	−6	119	122	+ 3	90	89	−1
A. B. 33	Functional G.I. 1.99	1+	15	76	75	−1	73	60	−13	79	75	−5
H. H. 33	Asthma 1.49	1+	15	89	81	−8	50	49	− 1	73	70	−3
Mean age, 38	Mean	Mean	12.2	87.1	81.6	−5.5	72.3	62.7	− 9.6	87.8	86.7	−1.1
	S.A.,	S.E.	3.13	3.17	2.75	1.10	9.80	12.1	3.76	5.34	5.82	0.91
Range, 30–58	1.76	S.D.	7.68	7.76	6.74	2.69	24.10	29.7	9.22	13.08	14.25	2.24
Statistical comparison with control series		p	>0.2 <0.3	>0.5	>0.5	0.1	>0.5	>0.5	>0.5	>0.5	>0.4 <0.5	>0.05 <0.1

and NSKP as the 1955 series during the octanoate infusion. In both the control and octanoate periods, mean EHBF, SPO_2 and BSP clearance did not differ significantly from the 1955 subjects.

Cortisone-hypoglycemic series (Table V)

Seven subjects were given 300 mg. cortisone acetate orally at 5 a.m. on the experimental day. Subsequently, they were made hypoglycemic at 7 a.m. and were studied in a manner exactly similar to the "posthypoglycemic series" above. Hypoglycemia was also attained in each of these subjects, the mean lowest blood sugar level for the group being 32.7 ± 2.31 mg. per cent. Due to technical difficulties only a "control period" study was made in Subject D.M.

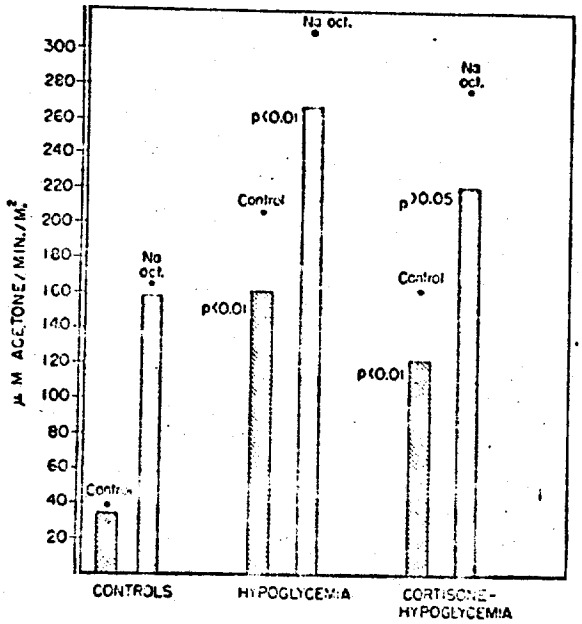

FIG. 1. NET SPLANCHNIC KETONE PRODUCTION BEFORE AND DURING SODIUM OCTANOATE INFUSION IN CONTROL, POSTHYPOGLYCEMIC AND CORTISONE-HYPOGLYCEMIC SERIES OF SUBJECTS (MEAN PLUS OR MINUS STANDARD ERROR)

The p values indicate the statistical comparison between the experimental and control series.

Compared to the posthypoglycemic group, cortisone administered two hours prior to the induction of hypoglycemia did not significantly alter the mean value of any of the measured parameters of carbohydrate or ketone body metabolism. However, closer examination of the ketone data in Table V reveals a remarkable variation of blood ketone levels and NSKP in the individual subjects included in this group. Although comparable degrees of hypoglycemia were attained in each of the seven individuals, Subjects C.P., E.K. and C.F. had the low basal NSKP levels of 37, 41 and 18 μM per minute per M.², as compared to the mean of 160.7 ± 46 in the posthypoglycemic group. On the other hand, Subjects E.H. and L.W. varied in the opposite direction with basal NSKP of 299 and 221 μM per minute per M.². Blood ketone levels revealed a parallel individual variation. Thus, although the mean data in this group indicate that cortisone pretreatment does not affect the ketogenic response to hypoglycemia, the considerable individual variation makes one accept this interpretation with caution.

This group did differ significantly from both the 1955 and posthypoglycemic series in one important respect, the presence of an elevated EHBF in the control period. The mean EHBF in this group was 1,019 ± 71.92 ml. per minute per M.², compared with 790 ± 38 in the 1955 series and 831 ± 33.4 per minute per M.² in the posthypoglycemic series ($p < 0.01$ and < 0.05). This finding is considered to be a direct or indirect effect of cortisone and is consistent with the previously reported data of Myers and Taylor demonstrating a 43 per cent increase in EHBF following cortisone therapy (5). This effect was partially obscured during the octanoate infusion when there was a fall in EHBF in all three groups.

DISCUSSION

In analyzing the data obtained by this technique it must be kept in mind that we are estimating net *splanchnic* ketone body production. While the viscera and mesentery drained by the portal vein are not the sources of ketone bodies *per se*, the mesenteric adipose tissue unquestionably contributes ketone body precursors as nonesterified fatty acids to the liver. Thus changes in NSKP as herein defined may be determined both by alterations in adipose tissue fatty acid release as well as true hepatic ketogenesis.

The data show that glucose added to an intravenous octanoate infusion significantly reduces the splanchnic ketone production observed when the latter alone is infused in man. The addition of insulin to the glucose-octanoate infusion did not alter the magnitude of this response. This effect of glucose is consistent with the large body of experimental evidence, recently reviewed in detail by Campbell and Best (6), suggesting that carbohydrate exerts its antiketogenic effect by suppressing ketone body production by the liver rather than by influencing peripheral tissue utilization of ketone bodies.

From our data it cannot be determined whether glucose affected the utilization of administered octanoate or simply inhibited endogenous ketone production from fatty acids derived from the liver itself or entering the liver from other sources. Observations by other workers support the conclusion that both mechanisms were influenced to some extent. Dole has clearly shown a sharp decrease

TABLE V

Metabolic studies three hours after insulin hypoglycemia in cortisone pretreated subjects

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.–F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.–F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs. C. P. 33	Neurosis 1.98	1+	22.2	25.8	3.6	58.5	77.0	18.5	+36.3	+51.2	+14.9
E. K. 29	Neurosis 2.12	1+	12.2	18.2	6.0	23.5	30.8	7.3	+11.3	+12.6	+ 1.3
E. H. 25	Functional G.I. 1.56	1+	67.0	93.0	26.0	122.3	163.3	41.0	+55.3	+70.3	+15.0
W. G. 31	Dermatitis 1.75	1+	49.3	67.0	17.7	94.0	118.2	24.2	+44.7	+51.2	+ 6.5
L. W. 41	Back pain 1.63	1+	52.2	74.0	21.8	93.3	118.3	25.0	+41.1	+44.3	+ 3.2
C. F. [illegible]	Functional G.I. 1.82	2+	12.5	13.2	0.7	38.0	54.8	16.8	+25.5	+41.6	+16.1
[illegible]. 34	Neurosis 2.00	1+	11.0	18.7	7.7						
Mean age, 32 Range, 25–41	Mean S.A., 1.84	Mean S.E. S.D.	32.3 8.85 23.0	44.3 12.58 37.70	12.0 3.77 9.8	71.6 15.44 37.83	93.7 7.73 18.94	22.1 4.58 11.22	+35.7 6.31 15.46	+45.2 6.26 15.35	+ 9.50 2.71 6.63
Statistical comparison with control series		p	<0.01	<0.01	<0.01	<0.01	<0.01	>0.5	>0.1 <0.2	>0.5	<0.01
Statistical comparison with hypoglycemic series		p	>0.5	>0.4 <0.5	>0.2 <0.3	>0.5	>0.4 <0.5	>0.1 <0.2	>0.5	>0.5	>0.2 <0.3

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs. C. P. 33	Neurosis 1.98	1+	1,090	1,164	+ 74	37	206	+169	36	41	+5
E. K. 29	Neurosis 2.12	1+	712	670	− 42	41	49	+ 8	42	33	−9
E. H. 25	Functional G.I. 1.56	1+	1,150	1,098	− 52	299	463	+164	34	43	+9
W. G. [illegible]	Dermatitis 1.75	1+	814	687	−127	144	170	+ 26	30	25	−5
[illegible]	Back pain 1.63	1+	1,015	1,071	+ 56	221	268	+ 47	32	40	+8
C. F. 29 [illegible]	Functional G.I. 1.82	2+	1,202	1,019	−183	18	170	+152	40	43	+3

TABLE V—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs. D. M. 34	Neurosis 2.00	1+	1,150			88			40		
Mean age, 32	Mean S.A., 1.84	Mean S.E. S.D.	1,019	951	− 46	121.1	221.0	+ 94.3	36.3	37.5	+1.83
			71.92	83.41	40.85	40.77	56.32	30.61	1.75	2.66	2.71
Range, 25–41			187.0	216.6	100.1	106.0	138.0	75.0	4.55	6.51	6.63
Statistical comparison with control series		p	<0.01	<0.01	>0.5	<0.01	>0.05 <0.1	>0.1 <0.2	>0.5	>0.3 <0.4	>0.4 <0.5
Statistical comparison with hypoglycemic series		p	>0.02 <0.05	>0.1 <0.2	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones %	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ*	BSP clearance % Control	During sod. oct.	Δ
yrs. C. P. 33	Neurosis 1.98	1+	22	102	92	−10	30	76	+46	80	79	−1
E. K. 29	Neurosis 2.12	1+	1	103	96	− 7	154	61	−93	82	75	−7
E. H. 25	Functional G.I. 1.56	1+	17	78	72	− 6	104	52	−52	71	71	0
W. G. 31	Dermatitis 1.75	1+	3	100	87	−13	95	44	−51	60	59	−1
L. W. 41	Back pain 1.63	1+	5	102	96	− 6	66	58	− 8	75	72	−3
C. F. 29	Functional G.I. 1.82	2+	18	97	95	− 2	36	31	− 5	91	86	−5
D. M. 34	Neurosis 2.00	1+		100			121			60		
Mean age, 32	Mean S.A., 1.84	Mean S.E. S.D.	11.0	97.4	89.7	− 5.3	86.6	53.6	−27.2	74.1	73.7	−2.8
			3.67	3.38	3.47	2.74	17.38	6.28	19.76	4.45	3.68	1.11
Range, 25–41			9.0	8.79	8.49	6.71	45.2	15.38	48.4	11.57	9.03	2.72
Statistical comparison with control series		p	>0.1 <0.2	<0.01	>0.02 <0.05	>0.2 <0.3	>0.1 <0.2	>0.2 <0.3	>0.1 <0.2	>0.05 <0.1	>0.1 <0.2	>0.5
Statistical comparison with hypoglycemic series		p	>0.5	>0.05 <0.1	>0.1 <0.2	>0.5	>0.5	>0.5	>0.4 <0.5	>0.05 <0.1	>0.05 <0.1	>0.2 <0.3

in plasma nonesterified fatty acids (NEFA) after glucose or insulin administration in man (7). This has been confirmed by Gordon and Cherkes (8). More recent observations by Gordon suggest that glucose and insulin decrease the liberation of NEFA from depot fat, leading to a decline in blood NEFA levels and finally to a cessation of extraction by other tissues (9). It is of particular interest that in three of his subjects studied by hepatic catheterization, positive arterial-hepatic venous blood NEFA differences were abolished by glucose administration. On the other hand, the *in vitro* studies of Weinhouse, Millington and Friedman (10) and Lossow, Brown and Chaikoff (11) have demonstrated that glucose prefeeding significantly reduced the conversion of labeled fatty acid substrates to both CO_2 and acetoacetate by liver slices. Furthermore, Lossow and Chaikoff (12) have observed that carbohydrate feeding significantly lowered the conversion of C^{14} of [illegible] injected fatty acids to CO_2 in intact rats. The effect was far more pronounced in the case of longer-chain fatty acids. However, it should be noted that the longer-chain fatty acids were administered in their experiments as unphysiological, complex emulsions in olive oil, whereas the shorter-chain acids such as octanoate were given as the sodium salt. In similar experiments recently reported by McCalla, Gates and Gordon (13) C^{14} labeled sodium palmitate was administered in the form of a serum albumin-bound complex ion which is believed to be the chemical form of endogenously circulating NEFA. Again, there was a marked inhibition of the conversion of intravenously administered palmitate to CO_2 in the carbohydrate fed intact rat, indicating the occurrence of a block in fatty acid oxidation *per se* at the cellular level. Current evidence suggests that endogenous NEFA are composed almost exclusively of these responsive longer-chain fatty acids (14). Since the carbohydrate sparing of shorter-chain fatty acid (octanoate) oxidation is minimal, we believe that the major carbohydrate effect in our experiments was mediated by a suppression of endogenous fatty acid mobilization or catabolism. In this regard it is noteworthy that the NSKP during the glucose-octanoate infusion experiments was roughly equivalent to the NSKP obtained during the administration of octanoate alone minus the endogenous control period NSKP.

Other data reported herein reveal that hypoglycemia is followed in three to four hours by elevated peripheral venous blood ketone levels, confirming the earlier reports of Collip (15) and Somogyi (16). The basis for this ketonemia in man now seems clearly demonstrated in the hepatic catheterization data in the six subjects which showed a 4.7-fold increase in net splanchnic ketone production three hours after hypoglycemia. This observation lends experimental support to the clinical experience that insulin hypoglycemia may initiate ketosis in the diabetic patient. Since the basal levels of splanchnic ketone production were markedly increased in the posthypoglycemic state, it was at first somewhat surprising to find that octanoate infusion in this group simply induced a net rise in ketone production quantitatively similar to that in the control subjects. This suggests that the ability to utilize the exogenous octanoate for ketone body synthesis is neither augmented nor decreased after hypoglycemia, but rather that the high basal level of ketogenesis is a result either of accelerated utilization of hepatic fatty acids or reflects an increased release of fatty acids from adipose tissue and their delivery to the liver where ketogenesis occurs. Support for the latter concept might be obtained by a study of blood nonesterified fatty acid levels after hypoglycemia, but such data are not recorded in the current literature. The basic biochemical events leading to ketosis in the posthypoglycemic state and their endocrine control have recently been reviewed in detail by one of us (17). The present study throws no light on the endocrine mechanisms involved in the ketonemia consequent to insulin hypoglycemia. However, concurrent studies with rats, to be published elsewhere (18), have shown that this ketosis still occurs in the absence of the pituitary, adrenal cortex and adrenal medulla, and hence hormones from none of these glands are essential. Once ketosis has developed it may be promptly abolished by the administration of a second dose of insulin despite continued hypoglycemia. In fasting hypophysectomized-adrenalectomized rats exhibiting marked hypoglycemia (35 mg. per cent) and ketonemia (258 μM per cent), administration of as little as 0.005 unit of insulin promptly restores blood ketone levels to normal without any further fall in the blood sugar. These data suggest that a relative deficiency in in-

sulin secretion during hypoglycemia may be the single most important hormonal factor contributing to the development of ketosis. It is interesting in this regard that tolbutamide, which reputedly stimulates insulin secretion, is also highly effective in depressing ketonemia during fasting and hypoglycemia (19).

Finally our data show that cortisone pretreatment did not significantly inhibit the elevation in basal net splanchnic ketone production in the posthypoglycemic state. We have pointed out that this interpretation must be accepted cautiously in view of the greater individual variations in this group. However, it is consistent with the recent observation of Amatruda and Engel (18) that cortisone pretreatment does not inhibit the development of ketosis following hypoglycemia in the intact rat. In contrast, it has been shown both in man (20) and the rat (21) that cortisone does inhibit fasting ketosis. Two known metabolic actions of cortisone make it a likely antiketogenic agent in the more slowly developing ketosis of fasting. First, cortisone tends to shift the balance of fat metabolism toward a net accumulation of fat either by inhibiting fat breakdown or stimulating fat synthesis. The second is its action in accelerating carbohydrate production by gluconeogenesis in the liver and in regenerating citric acid cycle precursors from protein catabolism. The answer to the discrepancy in the effect of cortisone on ketosis, suppressing fasting but not modifying posthypoglycemic ketosis, probably resides primarily in the nature of the ketogenic stimulus. It seems likely that the metabolic adjustments rapidly brought forth by the intense stimulus of hypoglycemia outweigh the above-mentioned antiketogenic actions of cortisone, particularly in view of the timing of the present experiments.

An alternate explanation is suggested by the recent finding of Scow, Chernick and Guarco (22) that cortisone is ketogenic in the "totally" pancreatectomized hypophysectomized rat and that this ketosis is prevented by insulin. Cortisone thus may be antiketogenic only when it is capable of bringing about an enhanced rate of insulin secretion. In these terms the variable responses in our cortisone treated posthypoglycemic subjects would reflect variations in the capacity of the pancreas in each case to secrete insulin following suppression by exogenous insulin administration.

SUMMARY

1. Further observations on net splanchnic ketone production (NSKP) were made in metabolically normal human subjects before and after the intravenous infusion of sodium octanoate.

2. The addition of glucose or glucose with insulin to the octanoate infusion significantly lowered the net rise in NSKP noted when octanoate alone was infused.

3. Ketosis in the peripheral blood was observed three hours after the induction of insulin hypoglycemia. While at this time basal NSKP was 4.7-fold greater than in the control subjects, the utilization of infused octanoate was unaffected.

4. Cortisone pretreatment failed to suppress the rise in peripheral blood ketone levels and NSKP occurring in the posthypoglycemic state.

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it may be concluded that a single species of binding site is predominant on transcortin both at 37° C and at 22° C.

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Regulation of Insulin Secretion by Short Chain Fatty Acids

EVIDENCE is accumulating that in some mammalian species there may be a direct effect of short chain fatty acids on the secretion of insulin from the pancreas. Thus it has been shown that octanoate causes a marked release of insulin from slices of rat pancreas[1]. Furthermore, butyrate was found to evoke a rapid and sustained elevation of serum insulin when infused into adult sheep[2]. These results contrast with the lack of effect on insulin secretion when longer chain fatty acids, such as palmitate, are incubated with pancreas slices[3].

With the development of methods for the isolation of islets of Langerhans in large numbers[4,5] it has become possible to study the mechanism of insulin secretion by the measurement of concentrations of metabolic intermediates. We have measured intracellular concentrations of glucose-6-phosphate following incubation of rat islets with butyrate, octanoate and citrate. The results suggest that short chain fatty acids are important regulators of glucose-6-phosphate levels in pancreatic islet cells, and in this way they may influence insulin secretion.

Islets of Langerhans were isolated from pancreas by collagenase digestion of tissue taken from male albino Wistar rats which had been fasted overnight. The separated islets were divided into groups of ten and incubated in bicarbonate-buffered medium containing albumin together with either glucose, octanoate, butyrate or citrate. In each experiment, the effects of low and high glucose were compared with those of low glucose plus organic acid. After 30 min of incubation at 37° C the medium was sampled for determination of its insulin content by the immunoassay of Hales and Randle[6]. Rat insulin provided standards for the assay. The islets from each group of incubation flasks were then pooled in pointed centrifuge tubes, the medium removed and the islets quickly frozen to −76° C by immersion of the tube in a bath of solid carbon dioxide and ethyl alcohol. They were then subjected to sonication for 20 s, after the addition of perchloric acid (0·1 ml./ten islets). The supernatants from the perchloric extracts were then neutralized with potassium carbonate, before the estimation of glucose-6-phosphate by the enzymatic cycling procedure described by Lowry *et al.*[7]. This gave us reproducible results in good agreement with those already reported by Matchinsky[8] for rat islets. Similar concentrations of glucose-6-phosphate have been reported by Idahl and Hellman[9] for islets taken from obese hyperglycaemic mice, in which β cells constitute more than 90 per cent of the islet tissue.

The results obtained are shown in Table 1, which shows that butyrate, octanoate and citrate all stimulate the release of insulin from isolated rat islets of Langerhans, and that paralleling the secretion there is an increase in the intracellular concentrations of glucose-6-phosphate in the islets.

These results can be explained as an effect of short chain fatty acids on the intracellular accumulation of citrate which may then affect phosphofructokinase, resulting in an inhibition of glycolysis and accumulation of glucose-6-phosphate, as has been suggested by Randle *et al.* for other tissues[10]. Furthermore, the results do not support the idea that the release of insulin brought about by sugars is mediated by glycolysis, for, in the presence of fatty acids, glycolysis is inhibited, but rather they suggest that glucose has to be metabolized by some other pathway in order to stimulate insulin secretion. We have already indicated that the pentose phosphate pathway may be important in this respect[11].

Table 1. SECRETION OF INSULIN AND THE INTRACELLULAR CONCENTRATIONS OF GLUCOSE-6-PHOSPHATE IN ISOLATED RAT ISLETS OF LANGERHANS INCUBATED IN THE PRESENCE OF OCTANOATE, BUTYRATE AND CITRATE

Addition to medium	Insulin secretion (μ units/10 islets/min; mean ± S.D.)	Glucose-6-phosphate levels (μμmoles/10 islets; mean ± S.D.)
2·5 mM glucose	18 ± 4 (20)	25·5 ± 7·0 (8)
20·0 mM glucose	39 ± 6 (20)	59·5 ± 10·0 (8)
2·5 mM glucose + 5·0 mM sodium citrate	37 ± 5 (20)	49·0 ± 12·0 (8)
2·5 mM glucose	18 ± 4 (20)	26·5 ± 5·0 (8)
20·0 mM glucose	38 ± 5 (20)	65·5 ± 11·0 (8)
2·5 mM glucose + 5·0 mM sodium butyrate	33 ± 4 (20)	47·5 ± 10·0 (8)
2·5 mM glucose	19 ± 3 (20)	22·5 ± 5·0 (4)
20·0 mM glucose	40 ± 5 (20)	51·0 ± 5·0 (4)
2·5 mM glucose + 5·0 mM sodium octanoate	38 ± 4 (20)	48·5 ± 7·0 (4)

It is not known whether longer chain fatty acids can also exercise a regulatory effect by this means, because so far it has been difficult to obtain effects of albumin-bound long chain fatty acids on insulin secretion. In addition, ketone bodies have also not been shown to be effective in releasing insulin in most species, whether used *in vitro*[7,11] or *in vivo*[12]. Thus we found that 5 mM β-hydroxybutyrate did not alter rates of secretion when incubated with isolated rat islets. Nevertheless, ketone bodies and long chain fatty acids may be important in modifying insulin release in certain other species, for Madison *et al.* have obtained effects of these agents in inducing insulin secretion in the dog[11,13]. By contrast, a much more consistent effect of short chain fatty acids on release of insulin has been obtained. Our results indicate that the "glucose fatty acid cycle"[10] may be operative as a means of metabolic regulation in pancreatic islet cells.

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Der Ketonkörperstoffwechsel im Hungerzustand und bei chronischer Unterernährung.

Von
Friedrich Müller.

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Mit 7 Textabbildungen.

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In den folgenden Untersuchungen wurden wir durch vielseitige Beobachtungen an Dystrophiekranken der Greifswalder Medizinischen Klinik in den ersten Nachkriegsjahren angeregt. Sie sind als die notwendige Ergänzung zu den Gülzowschen Arbeiten über den Kohlenhydratstoffwechsel bei Hunger und Unterernährung gedacht. Die wenigen über den Fettstoffwechsel bei Unterernährung vorliegenden Arbeiten befassen sich weniger mit Fragen des intermediären Stoffwechsels als mit solchen des Fettminimums und der Zufuhr gewisser ungesättigter Fettsäuren (Linol-, Linolen- und Arachidonsäure), die für Wachstum und Wohlbefinden auch des Menschen unerläßlich zu sein scheinen (Lang, Fleisch, Burr u. Burr, Evans u. Lepkovski), oder sie befassen sich mit pathologisch-anatomischen Feststellungen (Fettinfiltration in die atrophisch werdende Leber, Waterlow) und der Registrierung blutchemischer Daten (erniedrigte Werte für Blutfett und Lipoide: Feigl, Overzier, Gülzow). Besonders der niedrige Cholesterinspiegel gilt als Frühsymptom der einsetzenden Inanition (Gülzow).

Wichtiger ist, daß Berning bei dystrophischen Kranken in der Ödemphase beträchtliche Blutketonsteigerungen (im Mittel 11,3 mg-%) fand. Auffallenderweise bestand aber keine Ketonurie, wie sie nach unserer Erfahrung bei solch hohen Werten zu erwarten wäre. Berning hat diese Ketosen in Verbindung zu Gastrektasien und Magenatonien gebracht, wie sie im Verlauf von Dystrophien häufig auftreten können. Er glaubt, daß auch die Komaketosen der Diabetiker und die Narkose- und Hungerketose der frisch Operierten für die dabei so oft vorkommenden Magenatonien verantwortlich zu machen seien.

Demgegenüber haben wir mehrfach darauf hinweisen können, daß die Blutketonwerte von Dystrophikern niedrig liegen. Gülzow fand die Nüchternwerte sogar gelegentlich bis fast auf 0 abgesunken. Auch bei dystrophischen Diabetikern können sich nur im Gefolge deutlicher Stoffwechselentgleisungen höhere Blutketosen entwickeln (Müller). Wodurch sollte es auch zu einem vermehrten Ketonkörperanfall in der Dystrophie kommen? Die Hauptquelle der Ketonkörper, das Fettdepot, ist bis auf geringste, nicht mehr mobilisierbare Reste geschwunden.

Durch die Nahrung wird gleichfalls kaum Fett zugeführt. Und auch der exogene und endogene Eiweißumsatz ist äußerst reduziert.

Berning erklärt seine Dystrophikerketosen durch die gleichzeitige Glykogenverarmung der Leber. Derartige direkte Beziehungen zwischen Keto- und Leberglykogenmangel bestehen aber nicht (vgl. dazu auch Müller: Ketonkörperstoffwechsel). Näher liegt die Deutung, daß sich zur Unterernährung ein akuter Hungerzustand gesellte und so die Einschmelzung des Eiweißbestandes und vielleicht noch vorhandener Fettdepotreste ohne Kohlenhydratschutz erfolgte. Die schweren Enterocolitiden mit Resorptionsstörungen, unter denen so viele Dystrophiker gerade in der Ödemphase zu leiden haben, legen diese Zusammenhänge nahe.

Katsch wies vor einigen Jahren auf die interessante Beobachtung hin, daß bei den unterernährten Diabetikern der Nachkriegszeit solche mit einer konstanten, meist völlig therapieunzugänglichen Restacetonurie vermißt werden. Ebenso ist wohl nur durch den Mangel an ketoplastischen Depots zu erklären, daß das diabetische Koma in diesen Jahren ein verhältnismäßig seltenes Ereignis geblieben ist (Diskussion Katsch-Wenderoth). Nur in Bezirken katastrophalen Insulinmangels stieg die Komamorbidität und -letalität erschreckend an, aber auch hier nur im Jahre 1946, wie Knick u. Porstmann an Hand des Materials der Leipziger Diabeteszentrale nachweisen konnten, so lange nämlich den Diabetikern überhaupt noch einige Fett- und Eiweißreserven für den endogenen Umsatz zur Verfügung standen. Ein Jahr später sank die Komahäufigkeit bei den nunmehr latent oder manifest dystrophischen Diabetikern trotz weiterhin bestehender Insulinverknappung wieder auf das für die letzten Kriegsjahre übliche Niveau ab.

Beim völlig hungernden Menschen liegen die Verhältnisse anders als beim Dystrophiker. Hier kommt es nach Aufbrauch der relativ geringen Kohlenhydratreserven sehr bald zu einer beträchtlichen Ketose, die meist schon mit dem 3. Hungertag ihren Höhepunkt erreicht und dann mit dem Schwinden der Fett- und Eiweißreserven gegen Ende des Hungerzustandes allmählich wieder etwas abzusinken pflegt. Der hungernde Organismus ist hierbei durchaus einem ausschließlich mit Fett und Eiweiß ernährten gleichzusetzen.

Gewisse individuelle Schwankungen im Ausmaß der Ketose bestehen jedoch. So werden offenbar während des Hungerns manche adipöse Menschen, sicher wegen ihrer größeren Depots, leichter und ausgiebiger als magere ketotisch (Folin u. Denis a. u.). Auch unterscheiden sich die verschiedenen Tierarten in Hinsicht auf die Intensität der Ketose während des Hungerns beträchtlich. So bekommen Hunde und Katzen, wie wahrscheinlich alle Fleischfresser, keine Hungeracetonurie (Jost, Minkowski, Baer). Interessanterweise neigt auch das Schwein trotz seiner enormen Fettdepots nicht zur Ketose, was man wohl nur durch eine besondere lipophile Tendenz des Gewebes im v. Bergmannschen Sinne deuten kann.

Funktionelle Untersuchungen über den Fettstoffwechsel fehlen im absoluten Hunger genau so wie bei der chronischen Unterernährung. Wir haben deshalb versucht, mit Hilfe von Stoffwechselbelastungen neue Einblicke in den Ablauf der Fettverbrennung, insbesondere der Ketonkörperbildung und -beseitigung, im Hungerzustand und bei chronischer Unterernährung zu gewinnen.

Leider konnten in den Jahren, in denen uns so reichlich dystrophiekranke Menschen zur Verfügung standen, nur Einzeluntersuchungen durchgeführt werden, da die Beschaffung und insbesondere laufende Ergänzung mancher Chemikalien damals unmöglich war. Auf die viel wichtigeren Verlaufsbeobachtungen an Menschen mußten wir deshalb verzichten und waren gezwungen, mit Hunden zu experimentieren. Dabei war uns sehr wohl bewußt, daß der Hund wegen seiner nur

geringen Ketoseeignung für Fettstoffwechseluntersuchungen nicht sonderlich geeignet ist, und selbstverständlich deswegen auch Analogieschlüsse zum Fettstoffwechsel des Menschen nur mit Reserve gezogen werden dürfen. Wir wählten trotzdem für unsere Untersuchungen Hunde, da sie als Versuchstiere manche Vorzüge bieten. Wie kaum ein anderes Tier fügt sich der Hund bei richtiger Behandlung fast verständig in den experimentellen Gang der Untersuchungen ein. Abwehr- und Fesselungsreaktionen unterbleiben. Auch haben uns die Ergebnisse der Stoffwechselbelastungen während des absoluten Hungerns sehr bald gezeigt, daß wohl graduelle, jedoch keine prinzipiellen Unterschiede zwischen Mensch und Hund bestehen.

Als Stoffwechseltest wählten wir die i.v. Injektion isotonischer Lösungen von Natriumcapronat bzw. -caprylat. Auf die in mancher Hinsicht physiologischere peroraale Fettbelastung verzichteten wir mit Bedacht. Die sehr wechselnden Resorptionsbedingungen der für solche Belastungen immer großen Fettmengen hatten sich uns schon früher bei Untersuchungen an Diabetikern sehr störend bemerkbar gemacht. Um so mehr mußten sie bei der im Verlauf der Versuche sich entwickelnden Sub- und Afermentie hungernder und unterernährter Menschen und Hunde störend ins Gewicht fallen.

Capron- und Caprylsäure eignen sich von den niederen Fettsäuren für i.v. Belastungen besonders gut, da sie kaum oberflächenaktiv auf die Venenwand einwirken, die Injektionen somit immer völlig reizlos ohne Venenschmerz vertragen werden und das ketonämische Maximum später als bei Buttersäure in noch gut faßbarer Zeit nach der Injektion liegt (KRAINICK). Auch Hämolysen, wie sie bei Verwendung der Salze höherer Fettsäuren (gelegentlich schon bei Natriumcaprinatinjektionen) auftreten können, kommen nicht vor.

Für den Menschen wählten wir eine Dosierung von 40 cm³ der isotonischen Lösung, die in 4 min mit gleichbleibender Geschwindigkeit in die Cubitalvene injiziert wurden. Damit gelang es, beim gesunden Menschen regelmäßig eine kurzdauernde geringe Ketose zu erzeugen. Für den Hund von mittlerer Größe genügten schon 20 cm³, die in 2 min in die V. jugularis oder saphena eingespritzt wurden, um jedesmal eine deutliche Ketose zu bekommen. Capillare Blutentnahmen für die Blutketon- und Blutzuckerbestimmungen wurden gewöhnlich 2, 4, 6, 8, 10, 15, 30 und 60 min nach der Injektion der Capronsäure vorgenommen. Die Injektionen haben sämtliche Versuchspersonen gut vertragen. Nur wenige wurden während der Injektion etwas benommen und verspürten einen geringen Hustenreiz mit ranzigem Geschmack im Munde. Bei manchen Hunden setzte eine vertiefte Atmung ein. Sie waren gelegentlich bis zu 10 min nach der Injektion noch torkelig und benommen. Auffallenderweise gewöhnten sie sich aber nach einigen Belastungen an die Säure- bzw. Ketonüberschwemmung. Die anfangs sehr auffälligen Symptome verloren sich allmählich regelmäßig. Die Belastungen wurden jeweils morgens nüchtern 12 Std nach der letzten Mahlzeit bzw. der letzten Fütterung durchgeführt.

Der Ablauf der Capronsäureketose bei gesunden, nicht hungernden Menschen und Hunden.

Vor der Besprechung der Versuchsreihen ist es notwendig, den ketonämischen Normaltyp einer solchen Capronsäure- und Caprylsäurebelastung bei stoffwechselgesunden, nicht hungernden und nicht unterernährten Menschen und Hunden zu betrachten, um für den späteren Vergleich

die physiologischen Varianten des Testes zu kennen. Insgesamt wurden an 12 mischrassigen Hunden mittlerer Größe 33 „Normalbelastungen" gewonnen. Die Normalbelastungen an gesunden Menschen entsprechen in ihren Abläufen den von KRAINICK und später auch von mir veröffentlichten.

Auf einige Beobachtungen, die besonders von KRAINICK und mir bei früheren Untersuchungen zur normalen Capronsäureketose gemacht wurden, soll zunächst kurz hingewiesen werden.

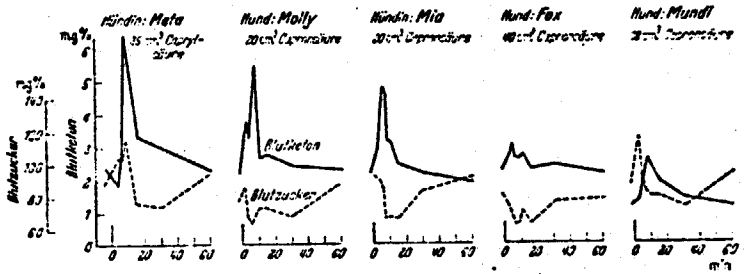

Abb. 1. Capryl- und Capronsäurebelastungen an stoffwechselgesunden, nicht hungernden Hunden.
Aus Raumgründen konnten von den 33 an verschiedensten Hunden gewonnenen Belastungen nur einige hier abgebildet werden. Die anderen stehen auf Wunsch zur Einsicht zur Verfügung.

KRAINICK hat 1940 mit $C_{(1)}$—$C_{(10)}$-Fettsäuren, die er i.v. als isotone Natriumseifenlösung injizierte, die β-Oxydation der Fettsäuren auch für den Menschen belegen können. Seitdem wurden ähnliche Fettsäurebelastungen zur Klärung von Fragen des Fett- und Ketonstoffwechsels öfter verwandt (MARKERS und Mitarbeiter, NATH u. BRAHMACHARI). Auch wir haben zu Untersuchungen über die diabetische Ketose und Ketolyse des Menschen und über den Diabetes des Hundes nach Alloxanvergiftung und Pankreatektomie die KRAINICKsche Fettsäurebelastung gewählt. Die Bildung der Ketonkörper aus den injizierten Fettsäuren im diabetischen Organismus geht praktisch mit der gleichen Geschwindigkeit vor sich wie im gesunden. Das ketonämische Maximum wird 11—12 min nach Beginn der Injektion erreicht, aber die Weiterverbrennung der Ketonkörper, die Ketolyse, ist abhängig vom jeweiligen diabetischen Stoffwechselzustand. Während beim stoffwechselgesunden Menschen und Tier die Blutketonwerte nach Erreichen des Gipfels sehr rasch, spätestens nach 15—30 min wieder zum Ausgangswert absinken, wird beim Diabetiker je nach seinem Stoffwechselzustand ein verzögerter Abfall oder sogar ein weiteres Ansteigen mit Übergang in Hungerketon beobachtet. Zur Illustration des eben Gesagten sind in der Abb. 2 die Capronsäurebelastungen eines Stoffwechselgesunden und eines Diabetikers einander gegenübergestellt.

Gleichzeitig mit den Ketonkörpern steigt bei Diabetikern wie Gesunden in den meisten Fällen auch der Blutzucker an. Es handelt sich dabei um eine immer kurzdauernde hyperglykämische Reaktion auf den Fettsäure- bzw. Ketosereiz infolge einer Glykogenmobilisation, die der reaktiven Hyperglykämie gleichzusetzen ist, die wir zusammen mit KRAINICK schon früher nach peroralen Fettbelastungen beobachteten. Sie ist seitdem auch von anderen Untersuchern beschrieben worden (MARKERS und Mitarbeiter). NATH u. BRAHMACHARI ist es sogar gelungen, durch

wochenlang fortgesetzte Fettsäureinjektionen den Blutzucker dauernd auf erhöhtem Niveau zu halten und schließlich einen Diabetes zu erzeugen.

Die Ketonämie nach Capronsäurebelastung beim gesunden Hund gleicht im wesentlichen der des Menschen. Nur sind die Ausschläge der Ketonkurven wegen der relativ größeren injizierten Fettsäuremengen beim Hunde höher. Die Gipfelwerte unterliegen allerdings von Hund zu Hund beträchtlichen Schwankungen, die sich nicht durch das nur wenig verschiedene Körpergewicht erklären lassen. Die Zusammenstellung einiger typischer, an Hunden gewonnener Normalbelastungen gibt dafür ein gutes Bild (Abb. 1, vgl. besonders Hündin Mia und Hund Fox). Dagegen zeigen die beim gleichen Hunde angestellten Belastungen auch gleichen Kurvenverlauf. Nur gelegentlich differieren die Gipfelwerte gering, da offenbar die Höchstwerte nicht immer erfaßt wurden. Einige Kurvenverläufe zeigen, wie rasch der Anstieg und der Abfall der Ketonwerte erfolgen kann, so daß eine zeitliche Verschiebung der Blutentnahme um nur 30 sec große Höhendifferenzen der Kurve zur Folge haben kann. Die ketonämischen Gipfel werden bei sämtlichen Belastungen innerhalb der ersten 10 min nach beendeter Injektion, bei vielen schon nach 4 und 5 min, erreicht. Meist fallen die Ketonwerte innerhalb von 10, spätestens von 15 min wieder auf den Normalwert ab, der wie beim Menschen zwischen 1,5 und 3 mg-% Gesamtketon liegt. Die Ergebnisse nach Capron- und Caprylsäurebelastung gleichen sich praktisch.

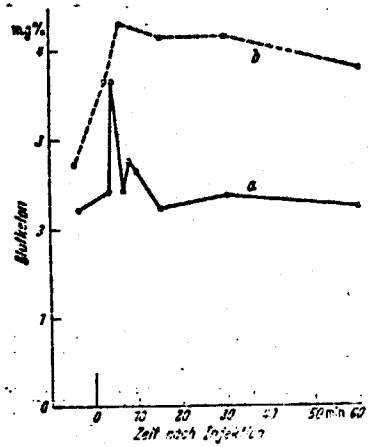

Abb. 2. Capronsäurebelastung bei einem Stoffwechselgesunden und einem Diabetiker. a) Ketonkurve des stoffwechselgesunden (Dr. F.). b) Ketonkurve des Diabetikers (E.F. Reg.-Nr. Garz 8937/17).

Zweifellos spiegelt sich in solchen Kurvenverläufen, *wie aktiv die Fette an den Verbrennungsprozessen auch des nicht hungernden und nicht glykogenverarmten Organismus teilhaben. Sie widerlegen eindeutig die bisherigen Vorstellungen von den „stoffwechselträgen, schwerverbrennbaren" Fetten.* Sie zeigen weiter, daß zumindest die exogene Ketonbildung auch beim Hunde ähnlich wie beim Menschen abläuft.

Die Blutzuckerkurven verlaufen weit weniger einheitlich als die Ketonkurven. Zwei Grundtypen herrschen jedoch vor. Der häufigere von beiden zeigt sofort nach der Fettsäureinjektion einen mehr oder weniger ausgeprägten, aber immer kurzdauernden Blutzuckeranstieg, dem ein

Abfall bis unter die Ausgangswerte folgt, gelegentlich bis zu ausgesprochen hypoglykämischen Werten (in einem Fall bis zu 45 mg-%). Bis zum Ende der Belastung nach 60 min hebt sich das Blutzuckerniveau wieder bis zum Ausgangswert oder auch beträchtlich darüber hinaus. Der zweite Typ läßt die primäre hyperglykämische Phase vermissen, die hypoglykämische setzt sofort nach der Injektion ein. Der folgende Wiederanstieg des Blutzuckers gleicht dem des erstbeschriebenen Kurventyps. Außer diesen beiden Formen kommen vereinzelt Varianten vor, die mehr oder minder deutliche Beziehungen zu einer der beiden Formen erkennen lassen.

Der seltenere zweite Kurventyp kommt wohl nur zustande, weil die hyperglykämische Reaktion so rasch abläuft, daß sie gelegentlich durch die Blutentnahme nicht erfaßt wird, oder weil sie durch sehr rasch einsetzende Verbrennungsprozesse ausgeglichen wird. Jedenfalls besteht keine Veranlassung, frühere Feststellungen über den primär hyperglykämischen Effekt durch Fettsäure bzw. Ketonreiz zu revidieren. 24 von den 33 Normalbelastungen am Hund lassen ihn mehr oder minder deutlich erkennen.

Die Deutung der Blutzuckerreaktion nach Fettsäureinjektion ist nicht schwierig. Sie ergibt sich zwanglos aus den bekannten und schon öfter erörterten Korrelationen, die bei der Keton- und Glucoseverbrennung angenommen werden müssen. Auf den Fettsäure- oder Ketonreiz hin wird, dem schnellen Ablauf der Ketogenese entsprechend, durch Glykogenolyse Glucose mobilisiert und zur Verbrennung bereitgestellt (primäre hyperglykämische Phase). Der sofort folgende Blutzuckerabfall bezeugt die aktivierte Verbrennung, durch die auch die Hyperketose rasch wieder zum Verschwinden gebracht wird. Dabei kann es zu einem übermäßigen Absinken des Blutzuckerniveaus im Sinne reaktiver Überleistungen (KATSCH) kommen.

Die Fettsäureverbrennung bei völligem Nahrungsentzug.

Aus den Verläufen der nach Capronsäureinjektionen gewonnenen Blutketon- und Blutzuckerkurven ergaben sich wichtige Aufschlüsse über den Ablauf der Fettsäureverbrennung am stoffwechselgesunden, normalgenährten Organismus. Bei den folgenden Untersuchungen legten wir uns die Frage vor, ob sich während und nach einer Periode völliger Nahrungskarenz die Blutketon- und Blutzuckerkurven der Capronsäurebelastungen ändern und ob gegebenenfalls daraus Rückschlüsse auf einen pathologischen Ablauf der Fettverbrennung gezogen werden können.

Dazu wurden nach genügend langer Vorbeobachtung 3 Hunde einer Hungerperiode von 17—22 Tagen unterworfen, während der sie im Stoffwechselgleichgewicht gehalten wurden und außer Leitungswasser keinerlei Nahrung zugeführt bekamen. Dabei kam es zu Gewichtsverlusten von $^1/_4$ bis $^1/_3$ des Ausgangsgewichtes. In der

sich bei allen 3 Hunden anschließenden Auffütterungsperiode wurde Wert auf besonderen Eiweiß-, Vitamin- und Kalkreichtum der Kost gelegt. Mit Caprylsäure bzw. Capronsäure wurden die Hunde in Abständen von jeweils wenigen Tagen belastet. Die genauen Daten sind aus den beigefügten Abbildungen zu ersehen. Anfangs glaubten wir, mindestens 30—40 cm³ Fettsäurelösung zu benötigen, um einen ausreichenden ketonämischen Effekt zu erzielen, jedoch sind 20 cm³ völlig ausreichend,

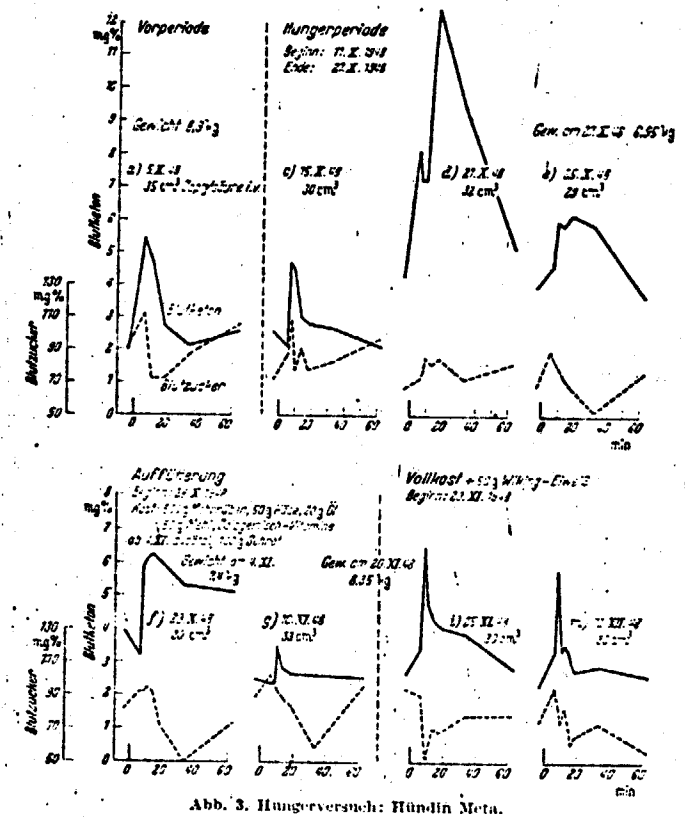

Abb. 3. Hungerversuch: Hündin Meta.

zumal es manchmal bei schlechten oder schon oft benutzten Venen schwierig war, größere Mengen als 20 cm³ mit der erforderlichen Geschwindigkeit von 10 cm³ min einzuspritzen.

Für entsprechende Versuche an Menschen stellten sich freundlicherweise einige Ärzte und Schwestern zur Verfügung. Hierbei mußten wir uns mit wesentlich kürzeren Hungerperioden begnügen, die gewöhnlich am 4. Hungertag endeten. Darnach konnte sofort ad libitum gegessen werden. Die Belastungen wurden jeweils morgens 8 Uhr durchgeführt und mit dem ersten Tag nach der Hungerperiode abgeschlossen. Einige Male wurde doppelt belastet, d. h. nach Ablauf der ersten Belastung wurden

nochmals 40 cm³ Capronsäurelösung injiziert und wie üblich Blutzucker und Blutketon im Capillarblut bestimmt. Weitere Einzelheiten sind aus den Versuchsprotokollen zu ersehen, die wir der besseren Anschaulichkeit wegen zum Teil graphisch aufgezeichnet haben (Abb. 4 und Tabelle 1).

Ergebnisse. Die Abb. 3 zeigt die Caprylsäurebelastungen des Hungerversuches an der Hündin Meta, die 17 Tage hungerte und deren Gewicht in dieser Zeit von 8,9 auf 6,95 kg zurückging. Die Kontrollbelastung der Vorperiode und auch die Belastung 5 Tage nach Beginn der Fastenperiode (Abb. 3a u. c)[1] bieten nichts Besonderes. Die Blutketonverläufe weichen von der Norm nicht ab.

Die Blutzuckerausgangswerte der Hungerbelastungen (Abb. 3c, d, e) liegen etwa 20 mg-% tiefer als die der Kontrollbelastungen. Der Blutzuckerverlaufstyp bleibt aber im wesentlichen erhalten. Nur am Ende der Hungerperiode (Belastung e) sinkt der Blutzucker während der Belastung bis zu dem hypoglykämischen Wert von fast 50 mg-% ab. Mit Beginn der Auffütterung hebt sich das Blutzuckernüchternniveau sofort wieder. Es bleibt aber noch lange eine ausgesprochene Neigung des Blutzuckers bestehen, bis zu Werten von 50 mg-% und darunter abzufallen.

Die Blutketonkurven dagegen verändern sich während des Hungerns deutlich. Schon ihre Ausgangswerte liegen über dem Normalbereich (Abb. 3d, e, f). Bei der am 10. Hungertag durchgeführten Belastung (Abb. 3d) steigt die Ketonkurve doppelt so hoch wie bei den Kontrollbelastungen an. Dabei wird das Maximum nur wenig später als normal erreicht, der Wiederabfall aber verzögert sich deutlich, wodurch die normalerweise schmale und schlanke Ketonzacke auffallend gespreizt wird. Noch 30 min nach Caprylsäureinjektion liegt der Ketonspiegel weit über dem Ausgangswert. Die gleiche Spreizung des Ketonkurvenverlaufs zeigen die letzte Belastung der Hungerperiode (e) und die einen Tag nach begonnener Auffütterung (f). Bei letzterer ist sogar nach 60 min der Ausgangswert noch nicht wieder erreicht. Den hohen Anstieg der Ketonkörper weisen diese Belastungen aber nicht mehr auf. Die übrigen Ketonkurven der Auffütterungsperiode verlaufen bis auf die der Belastungen i und m sehr flach und zeigen einen nur geringen ketotischen Ausschlag. Mit der letzten Belastung (m) dürfte der normale Kurventyp wieder erreicht worden sein.

Der zweite, sich über 22 Tage erstreckende Hungerversuch, bei dem die Hündin Minna über $^1/_3$ ihres Körpergewichtes verlor, und ihre Serumeiweißkörper (Refraktometerwerte) von 5,25 auf 3,5% absanken, läßt an den Ketonkurven prinzipiell die gleichen Veränderungen erkennen, wie sie eben geschildert wurden. Die Morgenketonwerte steigen

[1] Auf die Abbildungen einiger Belastungen dieses Versuches sowie sämtlicher Belastungen der beiden anderen Hungerhunde mußte wegen des zu großen Umfanges verzichtet werden. Sie können zur Einsicht angefordert werden.

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Tabelle 1. *Capronsäurebelastungen bei hungernden Menschen.* (40 cm³ isotonische Na-Capronatlösung i.v., Injektionsdauer 4 min.)

Nr.	Name	Datum	Blutzucker bzw. *Blutketon* in mg-% nüchtern	2 min	4	6	8	10	15	30	60
			nach beendeter Capronsäureinjektion								
1	Dr. R.	9. 1. 50, 8 Uhr	94	94	91	89	76	80	82	87	98
		Leerversuch	*2,11*	*2,83*	*3,64*	*2,55*	*2,69*	*2,55*	*2,11*	*1,83*	*1,97*
		10. 1. 50, 8 Uhr	64	63	63	59	59	61	66	68	72
		n. d. 1. Hungertag	*6,75*	*7,0*	*7,45*	*8,1*	*8,85*	*9,1*	*8,1*	*7,65*	*7,2*
		11. 1. 50, 8 Uhr	66	63	63	55	66	61	66	66	—
		n. d. 2. Hungertag	*5,4*	*8,35*	*8,85*	*8,35*	*9,35*	*7,2*	*6,55*	*7,45*	*7,0*
		12. 1. 50, 8 Uhr									
		1 Tag nach	84	82	80	85	82	78	87	85	72
		Hungerversuch	*1,97*	*2,38*	*2,69*	*3,0*	*2,55*	*1,97*	*1,97*	*1,42*	*1,69*
2	Dr. Fr.	17. 1. 50, 8 Uhr	91	92	94	96	103	94	98	—	98
		Leerversuch	*2,25*	*2,38*	*3,64*	*2,38*	*2,38*	*2,69*	*2,25*	*2,38*	*2,25*
		19. 1. 50, 8 Uhr	121	93	89	94	82	80	75	75	76
		n. d. 2. Hungertag	*1,65*	*5,8*	*5,95*	*8,1*	*7,85*	*8,85*	*9,35*	*7,65*	*6,35*
		20. 1. 50, 8 Uhr	79	82	86	79	79	73	82	57	77
		n. d. 3. Hungertag	*6,15*	*7,85*	*8,35*	*8,85*	*9,6*	*7,85*	*8,35*	*6,35*	*5,8*
3	G.	7. 2. 50, 8 Uhr	81	92	88	92	81	87	74	87	83
		Leerversuch	*2,55*	*3,3*	*3,16*	*3,47*	*3,6*	*2,83*	*2,69*	*2,38*	*2,25*
		Zweitbelastung		97	96	92	87	81	87	87	87
		im Anschluß an die		*3,0*	*2,83*	*2,83*	*2,55*	*2,69*	*2,55*	*2,25*	*2,38*
		vorhergehende									
		9. 2. 50, 8 Uhr	61	61	65	70	59	70	56	54	61
		n. d. 2. Hungertag	*9,95*	*10,2*	*10,8*	*11,1*	*9,85*	*7,85*	*8,85*	*7,65*	*7,85*
		Zweitbelastung		—	61	61	65	68	66	65	63
		im Anschluß an die		*8,1*	*8,35*	*8,35*	*8,1*	*7,65*	*9,6*	*8,6*	*7,85*
		vorhergehende									
		10. 2. 50, 8 Uhr	74	68	66	68	66	61	72	66	52
		n. d. 3. Hungertag	*10,2*	*10,8*	*11,4*	*11,7*	*12,7*	*11,1*	*10,8*	*10,5*	*10,8*
		8 g Traubenzucker									
		u. 50 g Rohrzucker	150'	210'	270'						
		p. os. i. Anschl. a. d.	120	129	127						
		Belastung	*7,0*	*4,3*	*2,69*						
4	Dr. G.	13. 2. 50, 8 Uhr	95	87	96	—	98	100	98	91	89
		Leerversuch	*2,25*	*2,54*	*2,55*	—	*2,69*	*2,83*	*2,69*	*2,38*	*2,11*
		Zweitbelastung		104	102	93	105	104	102	84	87
		i. Anschluß an die		*2,25*	*2,55*	*2,69*	*2,38*	*2,25*	*2,69*	*2,11*	*2,11*
		vorhergehende									
		14. 2. 50, 8 Uhr	96	92	—	103	96	97	83	97	94
		n. d. 1. Hungertag	*2,55*	*3,0*	*3,17*	*3,64*	*3,47*	*3,3*	*2,55*	*2,38*	*2,25*
		Zweitbelastung		96	76	103	99	88	92	92	90
		i. Anschluß an die		*2,83*	*3,0*	*3,16*	*3,3*	*2,69*	*2,83*	*2,38*	*2,11*
		vorhergehende									
		15. 2. 50, 8 Uhr	91	76	87	83	87	87	85	78	78
		n. d. 2. Hungertag	*4,65*	*5,95*	*7,2*	*7,0*	*8,1*	*6,55*	*5,95*	*5,55*	*5,2*
		16. 2. 50, 8 Uhr	65	78	74	81	72	74	78	72	72
		n. d. 3. Hungertag	*11,1*	*13,0*	*13,7*	*13,0*	*14,9*	*14,5*	*13,0*	*11,7*	*11,1*
		Zweitbelastung		69	74	71	67	67	71	72	74
		i. Anschluß an die		*14,1*	*14,9*	*16,7*	*13,7*	*14,5*	*13,0*	*11,4*	*10,2*
		vorhergehende									
		17. 2. 50, 8 Uhr									
		1. Tag nach	133	138	142	—	155	146	151	129	112
		Hungerversuch	*1,97*	*2,25*	*2,55*	—	*2,69*	*2,83*	*2,38*	*2,55*	*2,11*
		Zweitbelastung									
		i. Anschluß an die		110	107	105	107	98	103	105	100
		vorhergehende		*2,38*	*2,55*	*2,69*	*2,25*	*2,11*	*2,11*	*1,83*	*1,97*

mit Beginn der Hungerperiode an und erreichen etwa am 10. Hungertag ein Niveau von 4.5—5.5 mg-% das dann bis zum Ende des Hungerzustandes beibehalten wird. Bis auf eine zeigen sämtliche Belastungen während des Hungerzustandes die beschriebene typische Spreizung der Ketonzacke, die auch hier allein durch den verzögerten Abfall der Ketonwerte entsteht; denn das ketonämische Maximum wird immer ebenso schnell (spätestens 10 min nach beendeter Capronsäureinjektion) wie bei den Kontrollbelastungen der Vorperiode erreicht. Auch hier überragt

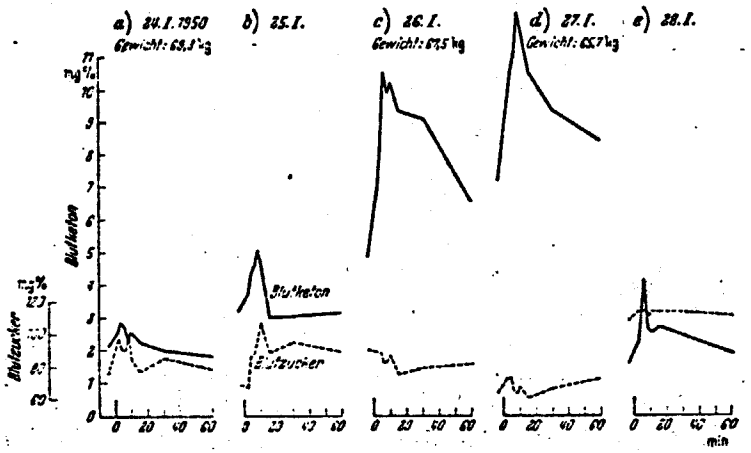

Abb. 4. Hungerversuch: Schwester K. B. Hungerperiode vom 24. 1. 50 bis zum Ende der Belastung am 27. 1. 50.

der ketonämische Gipfel einer Hungerbelastung die übrigen fast um das Doppelte. Bereits einen Tag nach Beginn der Wiederauffütterung beginnt die Ketonkurve mit einem völlig normalen Nüchternketonwert, fällt allerdings von schnell erreichter Höhe noch deutlich verzögert ab. Die anderen Belastungen der Nachperiode zeigen nichts Besonderes.

Die Blutzuckerkurven verlaufen nicht sehr charakteristisch. Immerhin sind die beim ersten Hungerversuch beschriebenen Veränderungen wenigstens angedeutet vorhanden, so die allgemeine Blutzuckerdepression und die Neigung zum Absinken auf hypoglykämische Werte während der Belastungen.

Die Belastungen des dritten Hungerversuches zeigen die in der Hungerphase auftretenden Veränderungen nicht so typisch. Sie sind nur angedeutet, so der verzögerte Abfall der Ketonwerte bei einigen Belastungen und der alle anderen deutlich überragende Ketongipfel am 13. Hungertag.

Von den 5 an hungernden Menschen durchgeführten Versuchsreihen wählten wir zur Abbildung nur eine aus (Abb. 4), die übrigen, deren

Ergebnisse den beiden abgebildeten weitgehend entsprechen, sind in der Tabelle 1 protokolliert.

Die Morgenketonwerte steigen während des Hungerns bis zum 4. Tage an. Der Blutzucker fällt dagegen regelmäßig etwas ab. Auch die Belastungsketosen steigern sich mit fortschreitendem Hunger beträchtlich. Dabei werden die Ketongipfel wieder mit derselben Geschwindigkeit (bei den abgebildeten Versuchen spätestens 8 min nach beendeter Injektion), jagelegentlich sogar früher als bei den Kontrollbelastungen erreicht. Der Wiederabfall der Ketonkörper verzögert sich jedoch zunehmend, und die daraus resultierende Spreizung der Ketonzacke prägt sich stärker aus. Bei einigen Belastungen liegen selbst die Endwerte nach 60 min noch weit über den Ausgangswerten (Abb. 4c u. d). Die abschließenden Belastungen, einen Tag nach dem Ende der Hungerperiode, zeigen gewöhnlich sofort wieder die gleichen Ketonkurven wie vor Beginn des Hungerns. Nur selten ist der ketonämische Ausschlag der Kurve noch etwas höher als normal (Abb. 4e).

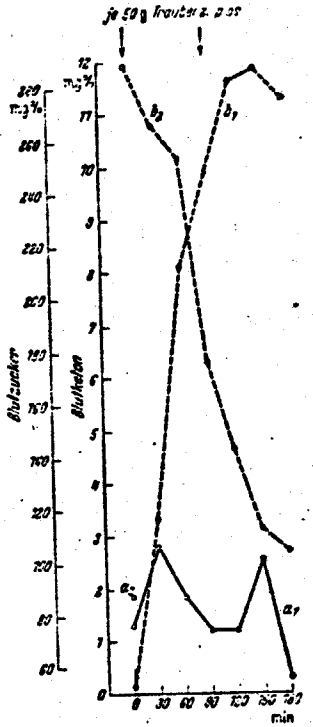

Abb. 5. Traugott-Staub Traubenzuckerdoppelbelastung nach einer Hungerperiode von 2 Tagen (Dr. R.). a_1: Blutzuckerkurve der Kontrollbelastung. a_2: Nüchternketonwert am Tage der Kontrollbelastung. b_1: Blutzuckerkurve der Bel. nach Hungerperiode. b_2: Blutketonkurve der Bel. nach Hungerperiode.

Auffallend hoch, wesentlich über dem üblichen Nüchterniveau, kann manchmal der Blutzucker dieser Schlußbelastungen liegen. So beginnt die Blutzuckerkurve einer solchen (Tabelle 1, Nr. 4) bei 132 mg-% und steigt bis zu 155 mg-% an. Sie fällt allerdings im weiteren Verlauf des Versuches bis auf normale Werte ab und reagiert auch auf die sich anschließende Zweitbelastung nicht wieder mit einem Anstieg. In einigen Fällen stellten wir am Ende des Hungerzustandes Traugott-Staubsche Glucosedoppelbelastungen an. Die Blutzuckerkurven solcher Belastungen verlaufen dann wie bei einem Diabetes mellitus. Sie zeigen einen sehr hohen Blutzuckeranstieg und einen noch höheren Blutzuckerzweitgipfel. Der Blutketonspiegel sinkt während der Belastung stetig ab und erreicht bald sein normales Niveau. Ein besonders schönes Beispiel bringt die Abb. 5.

Besprechung der Ergebnisse. Im folgenden wollen wir versuchen, den Ursachen der im Hungerzustand von der Norm abweichenden Veränderungen des Ketonstoffwechsels nachzugehen. Bei einem solchen Deutungsversuch läßt sich eine gewisse Spekulation nicht vermeiden. Zu viele, oft nicht zu trennende Stoffwechselvorgänge interferieren: Geschwindigkeit und Ausmaß der Verbrennungsprozesse, vermehrte Ketogenese und verminderte Ketolyse oder umgekehrt, Ablagerung der injizierten Capronsäure in den Fettdepots oder zusätzliche Mobilisation von Depotfett durch Reiz u. a.

Während des Hungerzustandes fallen drei typische, bei Hund und Mensch fast in gleicher Weise auftretenden Merkmale auf:

1. eine allgemeine *Zunahme der Blutketonkörper*,
2. eine *Verzögerung im Wiederabfall der Ketonwerte nach der Capronsäurebelastung*, schließlich
3. eine *beträchtliche Steigerung des ketonämischen Belastungseffektes* beim Menschen, zu der es beim Hunde nur in gewissen Phasen der Hungerperiode kommt.

Die allmähliche Zunahme der Blutketonkörper im Hunger bis zu einem erhöhten und dann bis zum Ende des Hungerzustandes meist gleichbleibenden Niveau ist seit langem bekannt. Von gewisser Bedeutung ist aber, daß sich auch bei manchen Hunden der Blutketonspiegel etwas hebt. Die Ausscheidung von Ketonkörpern durch die Nieren steigt dabei aber nicht so hoch an, daß die LEGALsche Probe positiv wird. Trotz dieser zweifellos nur geringen Ketose sinkt der RQ soweit ab, daß auch hier sicher nur Fett und Eiweiß verbrannt werden können (SNAPPER u. v. CREFELD). Daraus muß aber der Schluß gezogen werden, daß sich während des Hungerns beim Hunde Ketogenese und Ketolyse besser die Waage halten als beim Menschen und so eine Ketonstauung vermieden wird. Das kann daran liegen, daß sich der allgemeine Stoffwechselumsatz des Hundes während des Hungerns ausgiebiger und schneller als beim Menschen auf ein reduziertes Sparniveau einreguliert und sich etwa dem eines winterschlafenden Tieres nähert, oder daran, daß der Hund nur geringe Fettdepots zur Verfügung hat und deshalb sehr bald auf seinen Eiweißbestand als Energielieferanten angewiesen ist, bei dessen Abbau aber bekanntlich außer den Ketonkörpern auch reichlich Glucose als „antiketogene" Substanz anfällt. Die theoretische dritte Möglichkeit, daß der Hund ganz allgemein eine größere Fähigkeit zur Ketolyse besäße als der Mensch, findet an unseren Untersuchungen keine Stütze, da die an nicht hungernden Hunden gewonnenen Capronsäureketosen bei Berücksichtigung der verschiedenen Körpergewichte und Capronsäuremengen im Vergleich zu den an Menschen gewonnenen durchaus äquivalente Höhen erreichten.

Die für den Hungerzustand so charakteristische Abwandlung des Ketonkurventyps nach Capronsäurebelastung, der hohe Anstieg und das träge verzögerte Absinken der Ketose, läßt sich am zwanglosesten durch eine Hemmung der Ketolyse deuten. Die Ketogenese in der Leber ist dagegen offenbar nicht gestört, da selbst die hohen Ketonmaxima in der gleichen kurzen Zeit nach der Injektion wie bei Nichthungernden erreicht werden.

Die Hungerketonkurven stimmen mit denen, die wir früher an Diabetikern und diabetischen Hunden gewannen, völlig überein (Abb. 6), so daß sich die Frage aufdrängt, ob den gleichen Ketonkurventypen auch gleiche Störungen im Stoffwechsel entsprechen.

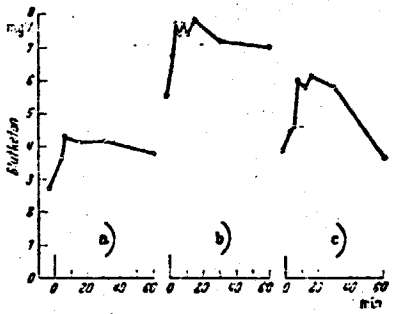

Abb. 6. Gegenüberstellung von Capronsäurebelastungen bei Diabetes mell. und im Hungerzustand. a) Ketonverlauf nach Belastung bei einer stoffwechselmäßig gut eingestellten Diabetikerin (EF Pat. Nr. Garz 8637 47). b) Belastung nach Pankreatektomie. Hündin Mia. c) Belastung nach 18tag. Hungerzustand. Hündin Meta.

Beiden Stoffwechselzuständen gemeinsam ist die Zuckernot der Gewebe; beim Diabetes infolge relativen oder absoluten Insulinmangels bei hohen Blutzuckerwerten, beim Hunger infolge effektiven Kohlenhydratmangels nach raschem Aufbrauch der geringen Reserven und offenbar nur zögernder Neubildung, wie man aus dem Absinken des Blutzuckerniveaus während des Hungerns auf fast hypoglykämische Werte schließen muß. Deshalb beseitigt auch schon geringe Zuckerzufuhr selbst hohe Hungerketosen sofort (Tabelle 1, Nr. 3).

Neben dem Kohlenhydratmangel kommen für die Ketolyschemmung im Hunger noch zwei weitere Faktoren in Betracht: *Ein allgemeiner Zell- und Gewebsschaden*, der mit fortschreitendem Hunger immer stärker zur Geltung kommen dürfte, *und ein relatives Insulindefizit* infolge allgemeiner Stoffwechseleinsparung und bei länger anhaltendem Hunger auch infolge Inaktivität und Hungeratrophie der Langerhans-Inseln. Außerdem gehört Insulin zu den Proteohormonen, zu deren Bildung hochwertige Eiweißkörper benötigt werden, die bei längerem Hunger fehlen. Insulinmangel und Gewebsschaden wirken etwa im gleichen Sinne hemmend auf den Stoffwechsel. Mit unserer Versuchsanordnung lassen sie sich anteilmäßig nicht trennen. Ihre wahrscheinliche Mitbeteiligung an den beschriebenen Hungerstoffwechselveränderungen ergibt sich aber aus folgenden Beobachtungen: 1. bleibt bei einigen Versuchen auch am Tage nach begonnener Wiederauffütterung der pathologische Ablauf der ketonämischen Belastungskurven zumindest angedeutet erhalten,

obwohl sich offenbar das Kohlenhydratdefizit ausgeglichen hat (normale oder erhöhte Blutzuckerlage!) (unter anderen Abb. 3f, 4e); 2. liegt auch der Blutzucker an diesem Belastungstag manchmal auffallend hoch und steigt zudem unter der Capronsäurebelastung noch weiter beträchtlich an (Abb. 4e, Tabelle 1, Nr. 4); 3. verlaufen Traugott-Staubsche Glucosebelastungen am Ende einer Hungerperiode ausgesprochen diabetisch (Abb. 5). Die beiden letzten Beobachtungen entsprechen ähnlichen GÜLZOWS und sind analog den Hungerglykosurien HOFMEISTERS und den Vaganten-Glykosurien HOPPE-SEYLERS zu werten.

Die Fettverbrennung bei chronischer Unterernährung.

Der Stoffwechsel des absolut hungernden Organismus erhält, wie wir gesehen haben, seine charakteristische Note durch den schnell einsetzenden Mangel an Kohlenhydraten. Auch die Neubildung von Zucker aus Eiweiß und vielleicht auch aus Fett bleibt ganz offenbar hinter dem Bedarf zurück. So wird der Hungerstoffwechsel im wesentlichen von den vorhandenen Fett- und Eiweißdepots bestritten. Bei der chronischen Unterernährung mit allmählich einsetzender Dystrophie liegen dagegen die Verhältnisse anders. Kohlenhydrate stehen hier trotz der Unterernährung immer reichlich zur Verfügung, häufig sogar reichlicher als bei normaler Kost, wenn auch die notwendige kalorische Gesamtenergie im allgemeinen durch sie nicht garantiert wird. Fett und Eiweiß werden aber mit der Nahrung kaum zugeführt, so daß es auch hier, wie bei absolutem Hunger, zu einer, allerdings langsameren Einschmelzung der Fett- und Eiweißbestände kommen muß, deren Verbrennung aber unter dem Schutz einer genügenden Kohlenhydratverbrennung vonstatten geht.

Für die vorliegenden Untersuchungen scheint uns deshalb die Beantwortung der Frage von besonderem Interesse, ob der Fettstoffwechsel auch bei chronischer Unterernährung trotz genügender Kohlenhydratzufuhr ähnlich von der Norm abweicht wie bei absolutem Hunger.

Ergebnisse. Leider war es nicht möglich, Verlaufsbeobachtungen an dystrophischen Menschen durchzuführen. Die Ergebnisse der während der Auffütterung an Dystrophikern gewonnenen Einzeluntersuchungen sind aus der Tabelle 2 zu ersehen. Sie bestätigen Bekanntes. Auch hier liegt das Nüchternketon der Dystrophiker niemals über, sondern häufig sogar unter der Norm. Ebenso lassen Adrenalin- und Capronsäurebelastungen keinerlei besondere Ketoseneigung erkennen (Tabelle 3 u. 4). Nur bei einer Belastung (Tabelle 3, Nr. 6) wird der normale Ketonbereich eben überschritten. Auch bei den beiden lipophilen Dystrophikerinnen (Tabelle 3, Nr. 4 u. 5) kommt es nach Adrenalininjektion nicht zur Ketose. Es ist daher möglich, daß die besondere lipophile Neigung dieser dystrophischen Fettsuchtsformen zu gewissen Phasen jegliche zusätzliche

Tabelle 2. *Nüchternketonwerte von Dystrophikern.*

Nr.	Name	Datum	Gewicht kg Grade %	Serumeiweiß %	Blutketon mg-%	Blutzucker mg-%	Bemerkungen
1	Anton K. Reg.-Nr. 1739/47	22. 11. 47	59,8 1,76	6,96	*1,53*	69	Wiederauffütterungsphase
2	Helmut D. Reg.-Nr. 2721/47	27. 11. 47	56,9 1,71	4,94	*1,14*	76	Nach Ödemausschwemmung
3	Otto H. Reg.-Nr. 2293/47	28. 11. 47	41,2 1,69	6,67	*2,38*	82	Durchfallphase mit Blutreinlicikung
4	Charlotte B. Reg.-Nr. 3101/47	6. 1. 48	50,8 1,80	4,94	*1,83*	96	Ödemphase
5	Kurt B. Reg.-Nr. 3446/47	13. 2. 48 17. 2. 48 21. 3. 48 6. 4. 48 13. 4. 48	52,5 1,68	4,94	*1,55* *1,28* *1,12* *1,55* *1,12*	70 73 63	Prädiabetes u. Inanition
6	Traute K. Reg.-Nr. 337/48	5. 4. 48 9. 4. 48	68,0 1,60	8,24	*1,12* *1,55*	88 93	Lipophile Dystrophie
7	Erna D. Reg.-Nr. 2748/48	2. 6. 48	75,0 1,56	6,86	*1,55*	74	Lipophile Dystrophie
8	Max Sch. Reg.-Nr. 3640/50	23. 11. 50	49,2 1,73	4,87	*2,55*	69	Nach Ödemausschwemmung
9	Bruno D. Reg.-Nr. 1160/49	1. 12. 49	55,7 1,65	5,83	*1,55*	72	Feuchte Dystrophie

Fettverbrennung verhindert, während ja sonst Fettsüchtige eher eine verstärkte Adrenalinketose aufweisen (MÜLLER).

Für die Untersuchungen an dystrophischen Hunden wählten wir drei mischrassige Tiere mittleren Gewichtes aus. 2 Hunde wurden nach Ausprägung schwersten dystrophischen Zustandes (nach 52 bzw. 38 Tagen

Tabelle 3. *Adrenalinbelastung bei Dystrophikern* (1 mg Adrenalin subcutan). (Die Daten für Gewicht, Größe und Serumeiweißkörper sind aus Tabelle 2 zu ersehen.)

Nr.	Name	Datum	Blutzucker bzw. *Blutketon* in mg-%, nüchtern	15 min	30	60	90	120	150	180
			nach Adrenalininjektion							
1	Anton K.	22. 11. 47	69	60	100	100	86	82	78	94
			1,83	*2,11*	*1,97*	*1,55*	*1,14*	*1,83*	*1,28*	*1,14*
2	Otto H.	28. 11. 47	82	86	100	135	128	89	89	71
			2,38	*2,69*	*2,83*	*2,83*	*2,55*	*2,25*	*2,25*	*2,11*
3	Kurt B.	17. 2. 48	73	68	82	111	110	108	92	83
			1,28	*1,69*	*1,55*	*1,42*	*1,42*	*1,55*	*1,69*	*1,42*
4	Traute K.	5. 4. 48	88	107	107	111	85	116	81	125
			1,42	*1,42*	*1,55*	*1,42*	*1,42*	*1,55*	*1,28*	*1,42*
5	Erna D.	2. 6. 48	74	88	84	77	68	83	52	77
			1,55	*2,11*	*2,83*	*2,25*	*1,55*	—	*2,11*	*2,69*
6	Max Sch.	23. 11. 50	69	74	76	83	81	78	76	67
			2,55	*2,83*	*2,83*	*3,00*	*3,3*	*3,16*	*2,83*	*2,55*

Tabelle 4. *Capronsäurebelastungen bei Dystrophikern* (50 cm³ Capronsäurelösung i.v. Injektionsdauer 4 min).

Nr.	Name	Datum	Blutzucker bzw. *Blutketon* in mg-%, nücht.	3 min	7	15	30	60	90
			nach Capronsäureinjektion						
1	Charlotte B.	6. 1. 48	96	105	103	103	62	82	96
			1,83	*1,55*	*1,55*	*1,83*	*2,11*	*1,83*	*1,83*
2	Helmut D.	27. 11. 47	76	71	69	80	94	94	91
			1,14	*1,38*	*1,42*	*1,69*	*1,38*	*1,38*	*1,69*

Unterernährung) durch Ausbluten getötet. Beide zeigten bei der Sektion eine ausgesprochene Fetteirrhose der Leber. Bei dem 3. Hund wurde eine Auffütterungsperiode angeschlossen, während der wir die Capronsäurebelastungen fortsetzten. Die Belastungen selbst wurden regelmäßig nüchtern 8 Uhr morgens durchgeführt. Einzelheiten der Versuchsanordnung, der Kost, der Daten über Gewicht usw. sind aus dem abgebildeten Protokoll (Abb. 7) ersichtlich[1].

[1] Aus Raumgründen konnten von den 23 während des Versuches angestellten Belastungen nur einzelne typische in der Abbildung dargestellt werden. Auch auf die graphische Wiedergabe der beiden anderen Dystrophieversuche mußte aus dem gleichen Grunde verzichtet werden. Die abgebildeten Protokolle stehen Interessenten zur Einsicht zur Verfügung.

Hund Molly (Abb. 7): An eine etwa einen Monat währende Vorperiode mit gewöhnlicher, ausreichend Eiweiß enthaltender Vollkost, an deren Ende der Hund 6,5 kg wog und einen Serumeiweißspiegel von 5,60% aufwies, schloß sich eine Unterernährungsperiode von 98 Tagen mit einer ausgesprochenen Mangelkost, bestehend aus 250 g Wruken, 25 g Mehl und 5 g Fett (20 g Kohlenhydrate, 6 g Eiweiß, 6,0 g Fett, 159 Cal.) an, in der das Gewicht des Hundes zunächst relativ schnell, später deutlich langsamer und z. T. ausgeglichen durch Wassereinlagerung bis auf 4,3 kg abnahm. Die Serumeiweißkörper sanken zunächst bis auf 3,3% ab, nahmen aber bis zum Ende der Dystrophieperiode, sicher infolge Bluteindickung, wieder bis

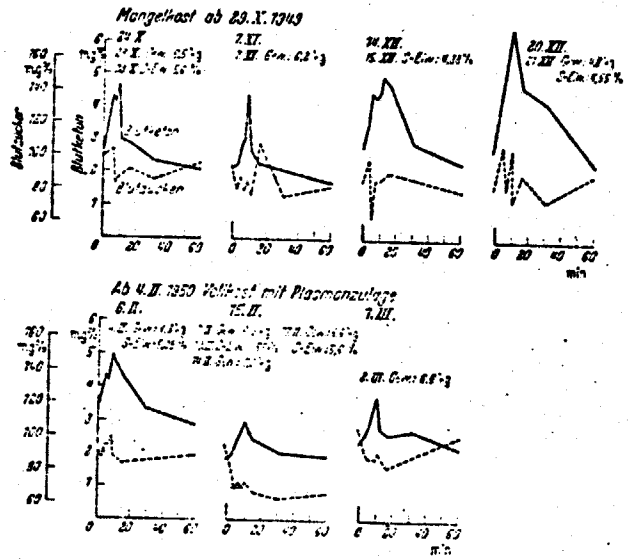

Abb. 7. Unterernährungsversuch Hund Molly. Ab 29. 10. 49 Mangelkost: 250 g Wruken, 25 g Mehl, 5 g Fett (20 g Kohlenhydrate, 6 g Eiweiß, 6 g Fett, insgesamt 159 Kalorien) Ab 4. 2. 50 Vollkost mit 50 g Plasmonzulage. Zu den Belastungen jeweils 20 cem isot. Na-Capronatlösung i. v.

zu 5,08% zu. Während der Auffütterung (Vollkost + Eiweißzulage von 50 g Plasmon) nahm der Hund rasch wieder an Gewicht zu und lagerte reichlich Fett ab. Er verblieb in diesem lipophilen Stadium, welches als überkompensierendes Regulationsgeschehen der lipophilen Dystrophie des Menschen völlig gleichzusetzen ist, bis zum Ende des Versuches nach etwa einem Monat. Auch das Gewicht des Hundes lag in dieser Phase bis Versuchsende höher als ausgangs.

Die Blutzuckerkurven verliefen während Unterernährung und Wiederauffütterung unauffällig. Nur bei 2 Belastungen zu Beginn der Unterernährungsperiode traten hypoglykämische Werte von fast 50 mg-% auf.

Die Ketonkurven dagegen zeigen dieselben Veränderungen, allerdings nicht so regelmäßig und gelegentlich nur angedeutet, wie wir sie bei den völlig hungernden Hunden beschrieben haben. Besonders bei unserem als

Beispiel ausgewählten Hund kommt mit fortschreitender Dystrophie die Verzögerung im Wiederabfall der Capronsäureketose und die daraus resultierende Spreizung der Ketonkurven deutlich zur Geltung. Nach Beginn der Wiederauffütterung gleicht sich die Spreizung des Ketonkurvenverlaufes sofort wieder aus. Ebenso wie bei den Hungerhunden fallen auch hier einzelne Belastungen mit besonders hohen Ketongipfelwerten auf, wie sie weder in den Vor- und Nachperioden, noch bei Belastungen an anderen nicht hungernden Hunden beobachtet wurden (vgl. Abb. 1). Bei fast sämtlichen Belastungen der Auffütterungsperiode dagegen, besonders während der lipophilen Ansatzphase dieses Hundes liegen die Ketongipfel auffallend niedrig.

Besprechung. Bei dystrophischen Hunden können ähnliche Ketolysehemmungen im Ablauf der Capronsäureketosen auftreten wie im absoluten Hunger und beim Diabetes mellitus. Ein grob rechnerischer Überschlag — unter Zugrundelegung der Shafferschen Fett- und Kohlenhydratrelation — ergibt sofort, daß genügend Kohlenhydrate mit der Nahrung zugeführt wurden, um die vollständige Verbrennung des einschmelzenden Depotfettes und Körpereiweißes zu garantieren. Der kalorische Index lag am Ende der Dystrophieperiode des Hundes Molly bei 37. Auch das Absinken des Blutzuckerniveaus konnte vermieden werden. *Ich halte deshalb den Schluß für erlaubt, daß die Hemmung der Ketolyse bei chronischer Unterernährung mit Dystrophie nicht durch Mangel an Kohlenhydraten wie bei absolutem Hunger, sondern vorwiegend durch ein Absinken der Insulinproduktion und durch Verschlechterung des Stoffwechselumsatzes infolge allgemeinen Zellschadens verursacht wird.* Die Frage, welchem von den beiden Faktoren, Insulinmangel oder Gewebsschädigung, dabei eine größere Bedeutung zukommt, bleibt solange müßig, bis es gelingt auch kleine Mengen von aktivem Insulin im Blut nachzuweisen. Dystrophische Menschen bekommen, wie wir oben schon sahen, nur deshalb keine Ketose, weil ihnen weder in der Nahrung noch in den Depots nennenswerte Mengen ketoplastischer Grundstoffe zur Verbrennung bereitstehen. *Die Ketolysehemmung in der Dystrophie kann erst nach Fettbelastung evident werden.*

Die geringen ketotischen Ausschläge der Capronsäurebelastungen in der Auffütterungsperiode finden ziemlich zwanglos durch die gleichzeitig vorhandene lipophile Tendenz des Fettgewebes ihre Deutung. Offenbar wird die injizierte Capronsäure gar nicht nennenswert verbrannt, sondern sofort in den Depots abgelagert. Ebenso muß das Ausbleiben jeglicher ketotischer Belastungsreaktion während der Auffütterung bei dystrophischen Menschen (Tabelle 3 u. 4) aufgefaßt werden.

Es sei nochmals auf die häufigen Parallelen hingewiesen, die sich bei Hunger und Unterernährung zwischen dem Stoffwechsel von Kohlenhydraten und von Fetten ergeben. *Der Stoffwechsel beider Stoffe zeigt bei absolutem Hunger und in der Dystrophie Veränderungen, die denen beim Diabetes mellitus weitgehend gleichen.* Nur das Verhältnis der jeweils auslösenden Faktoren ändert sich. Bei Diabetes stehen Insulinmangel und daraus sich ergebende Zuckernot der Gewebe im Vordergrund, ein sicher auch vorhandener Gewebsschaden tritt im allgemeinen zurück und kommt erst in der Stoffwechseldekompensation, im diabetischen Koma, zur Geltung (MÜLLER). Anders bei absolutem Hunger. Hier spielt fraglos der effektive Kohlenhydratmangel die Hauptrolle, und Insulindefizit und Gewebsschädigung sind demgegenüber belangloser. In der Dystrophie sind es jedoch gerade diese beiden Faktoren, die an der Fettverbrennungsstörung teilhaben.

Für den Kohlenhydratstoffwechsel sind die aufgezeigten Beziehungen zwischen Hunger, Unterernährung und Diabetes schon vor Jahrzehnten von HOFMEISTER (Hungerglykosurie bei unterernährten Hunden) und von HOPPE-SEYLER (Vagantenglykosurie) vermutet worden. In letzter Zeit ist besonders GÜLZOW dieser Frage in mehreren Arbeiten nachgegangen und hat die vermuteten Beziehungen gesichert. *Wir konnten zeigen, daß nicht nur die Kohlenhydrate, sondern auch die Fette, also der gesamte Energiestoffwechsel in gleicher Weise beim Diabetes mellitus, im absoluten Hunger und bei chronischer Unterernährung reagieren.*

Zusammenfassung.

Die Stoffwechselvorgänge im Hunger und in der Unterernährung wurden bisher im wesentlichen nur von den Kohlenhydraten und den Eiweißen her betrachtet. Wir haben versucht, mit Hilfe von Stoffwechselbelastungen neue Einblicke in den Ablauf auch der Fettverbrennung, der Ketogenese und Ketolyse, im akuten Hungerzustand und bei chronischer Unterernährung zu gewinnen.

Vorversuche mit Hilfe von Capronsäurebelastungen an stoffwechselgesunden, nicht hungernden Hunden und Menschen zeigten erneut, wie aktiv und rasch die Fette an den Verbrennungsprozessen auch des nicht hungernden und nicht glykogenverarmten Organismus teilhaben. Sie widerlegten eindeutig die bisherigen Vorstellungen von den „stoffwechselträgen und schwerverbrennbaren" Fetten.

Capronsäurebelastungen an akut hungernden Hunden und Menschen ergaben eine auffallende Verzögerung im Wiederabfall der Ketonwerte nach der Belastung, die sich am zwanglosesten nur durch eine Hemmung der Ketolyse deuten ließen. Außerdem zeigte sich besonders beim Menschen eine beträchtliche Steigerung des ketonämischen Effektes.

Auch an dystrophischen Hunden wurde die gleiche Ketolysehemmung im Ablauf der Capronsäureketose beobachtet, obwohl genügend Kohlenhydrate mit der Nahrung zugeführt wurden. Es wird deshalb der Schluß gezogen, daß die Hemmung der Ketolyse bei chronischer Unterernährung

mit Dystrophie nicht durch Mangel an Kohlenhydraten wie beim absoluten Hunger, sondern vorwiegend durch das Absinken der Insulinproduktion und durch Verschlechterung des Stoffwechselumsatzes infolge allgemeinen Zellschadens verursacht wird.

Sowohl bei absolutem Hunger als auch bei der Dystrophie haben sich damit Veränderungen im Fettstoffwechsel ergeben, die denen beim Diabetes mellitus weitgehend gleichen.

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Aus dem Physiologischen Institut
(Direktor: Prof. Dr. med. u.

Beitrag zur Frage der Bl
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Von

ANNEMARIE

Mit 5 Textabb

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Seitdem das tiefere Verständnis un
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Proceedings of the Montana Academy of Sciences 28: 51-57, 1968

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EFFECT OF FATTY ACIDS ON *SHIGELLA**

Mitsuru Nakamura and Marilyn Jo Zangar**

The mechanism of pathogenicity in *Shigella* has not yet been clearly elucidated. A number of variables appear to be important; namely, the host, the age of the host, the size of the infective dose, the "strain" and species of the etiologic agent, and the normal intestinal flora. The role of the normal intestinal flora in *Shigella* infections has been reviewed recently (5, 6). There is general agreement that the normal bacterial flora protects the animal against infection and disease due to the *Shigellae*.

Several workers reported that the growth of *Shigella* was inhibited when grown in a mixed culture with other bacteria (1, 4). The associated bacteria apparently produced some chemical or physical change in the environment that affected the growth of the pathogen.

The mechanisms of these interacting reactions become of interest in order to establish the specific role of the normal flora. Hentges reported that *Klebsiella* inhibited *Shigella* in mixed cultures (2). He suggested that the formic and acetic acids produced by *Klebsiella* were responsible for the inhibition of the *Shigella*.

The present investigation was undertaken to study the effects of several fatty acids on the *in vitro* growth of *S. sonnei* and the related species *Escherichia coli* as well as the enteropathogenic variant of *E. coli*. The effects of acetic, propionic, and octanoic acids were studied on *E. coli* B, *E. coli* OB E 0127:B8 (enteropathogenic), *S. sonnei* strain 9453, and *S. sonnei* strain 6761. The bacteria were maintained and exposed to fatty acids in the chemically defined medium (2). The pH of the media containing various concentrations of fatty acids ranging from 0 to 1.8 percent was adjusted to 7.0.

Standard plate counting techniques were used to enumerate the bacterial populations after incubation for 24 hours at 37° C. The activity of the fatty acids on the growth of

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** Undergraduate research participant in microbiology supported by grant GY-2547, Undergraduate Education in Science, National Science Foundation.

Cap. Acid

E. coli B is illustrated in Figure 1. Octanoic acid was highly toxic and a concentration of 0.3 percent essentially sterilized the culture. Acetic and propionic acids, after producing an initial partial inhibition, did not significantly reduce the growth of this organism even at 1.5 to 1.8 percent. Octanoic acid was the most toxic fatty acid for *E. coli* OB E 0127:B8 (Fig. 2). Acetic and propionic acids were less inhibitory to this strain.

The dose-response curves in Figure 3 and Figure 4 illustrate the activities of the fatty acids against *S. sonnei* strains 9453 and 6761. Octanoic acid was the most bactericidal of the fatty acids. Propionic acid was inhibitory to both strains, although there was a slight difference in the rate of inhibition. The lethal effect of propionic acid was exponential. Acetic acid was highly toxic to strain 6761 but relatively nontoxic to strain 9453 of *S. sonnei*. This is interpreted as due to strain difference in susceptibility to acetic acid.

The three fatty acids studied exerted different inhibitory patterns on the related bacteria studied. According to Hentges the inhibitory activity of acetic acid cannot be attributed to a hydrogen ion effect alone (3). However, the mechanisms by which these fatty acids exert their antibacterial effect are not understood.

Hentges has hypothesized that the fatty acids, particularly acetic acid, are inhibitory for *Shigella* in the intestine (2). It is possible that the fatty acids produced in the intestine by the normal flora inhibit the *Shigella* and thus reduce the possibilities of disease following infection. In view of the fact that our results suggest a considerable variation in susceptibility of *S. sonnei* to acetic acid, we would like to suggest that other mechanisms may operate, besides fatty acid toxicity, in the interaction between *Shigella* and the normal intestinal flora. Furthermore, the acetic acid produced in the intestine will also inhibit *E. coli*, resulting in a much more complex interacting system than merely the inhibition of *Shigella* by the products of another organism.

Currently we are studying the effects of other fatty acids on *Shigella*. We plan to study the effects of fatty acids on *Shigella-Escherichia* hybrids in order to determine if there are phylogenetic relationships among these organisms in relation to fatty acid sensitivity.

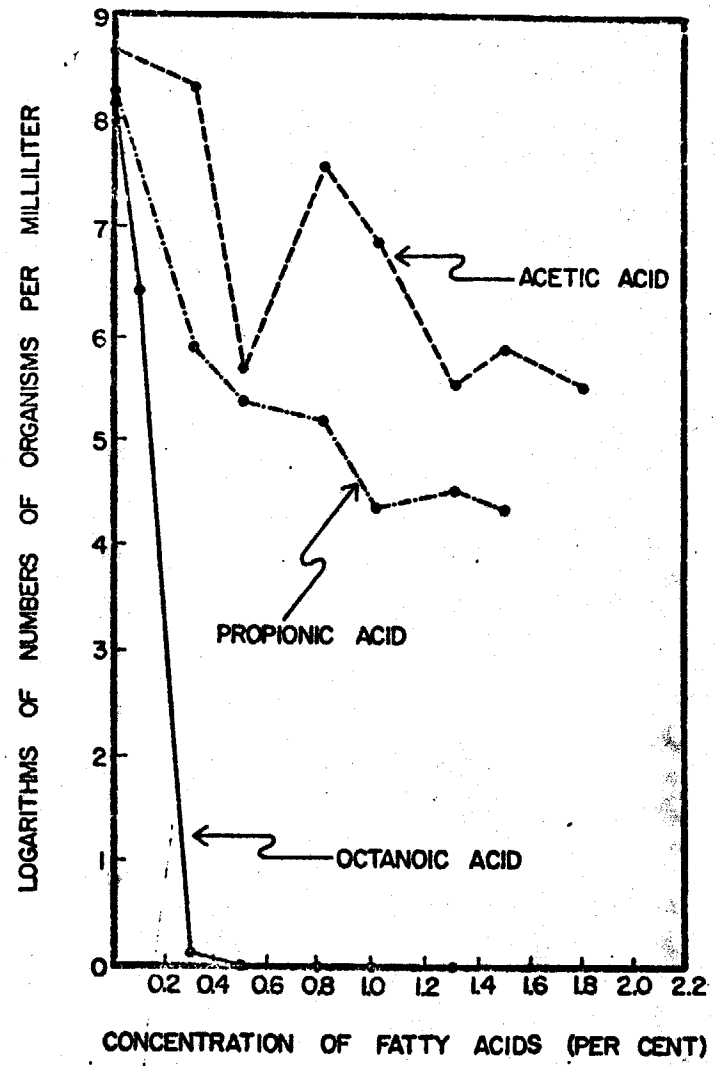

Figure 1. Effect of Acetic, Propionic, and Octanoic Acids on *Escherichia coli* B.

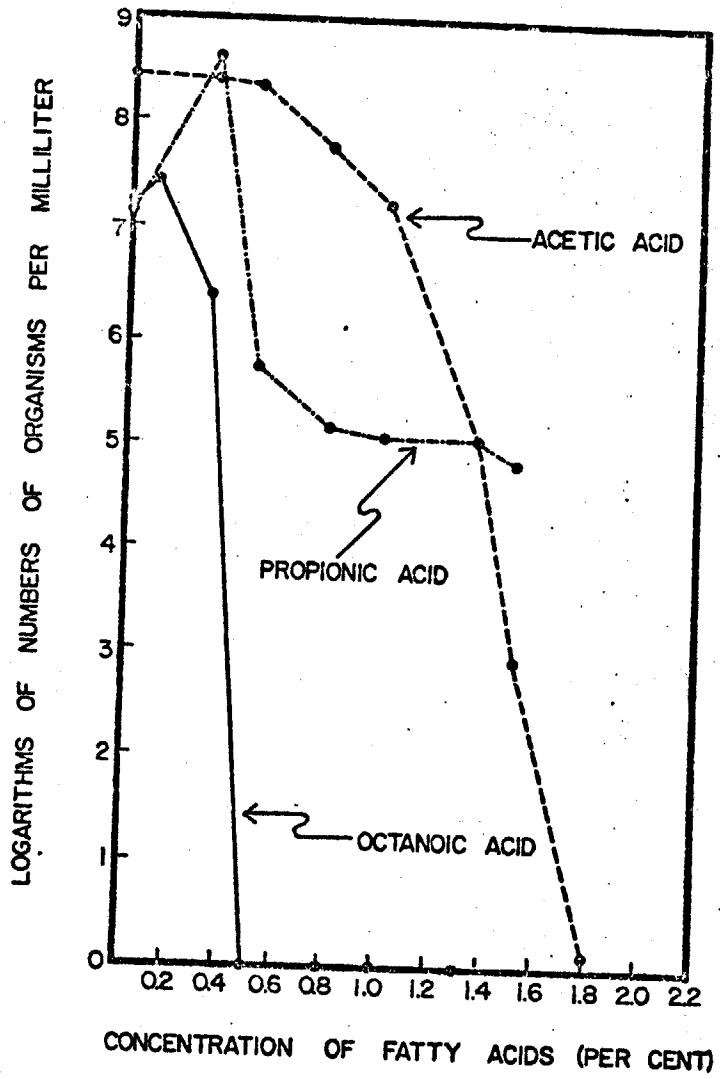

Figure 2. Effect of Acetic, Propionic, and Octanoic Acids on *Escherichia coli* OB E 0127:B8.

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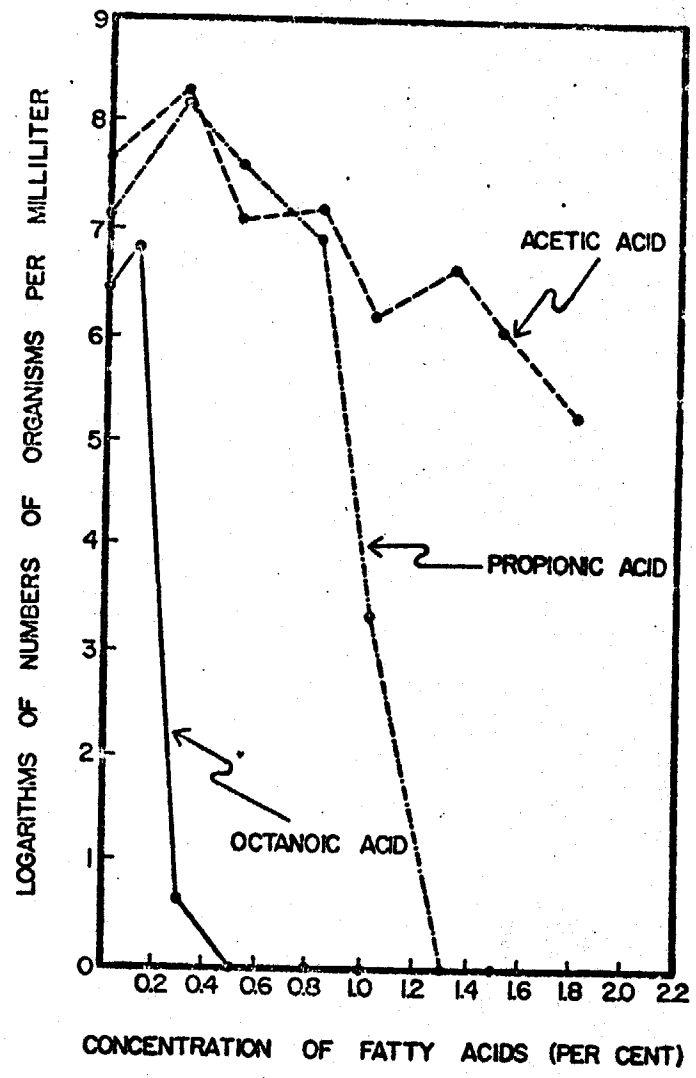

Figure 3. Effect of Acetic, Propionic, and Octanoic Acids on *Shigella sonnei* 9453.

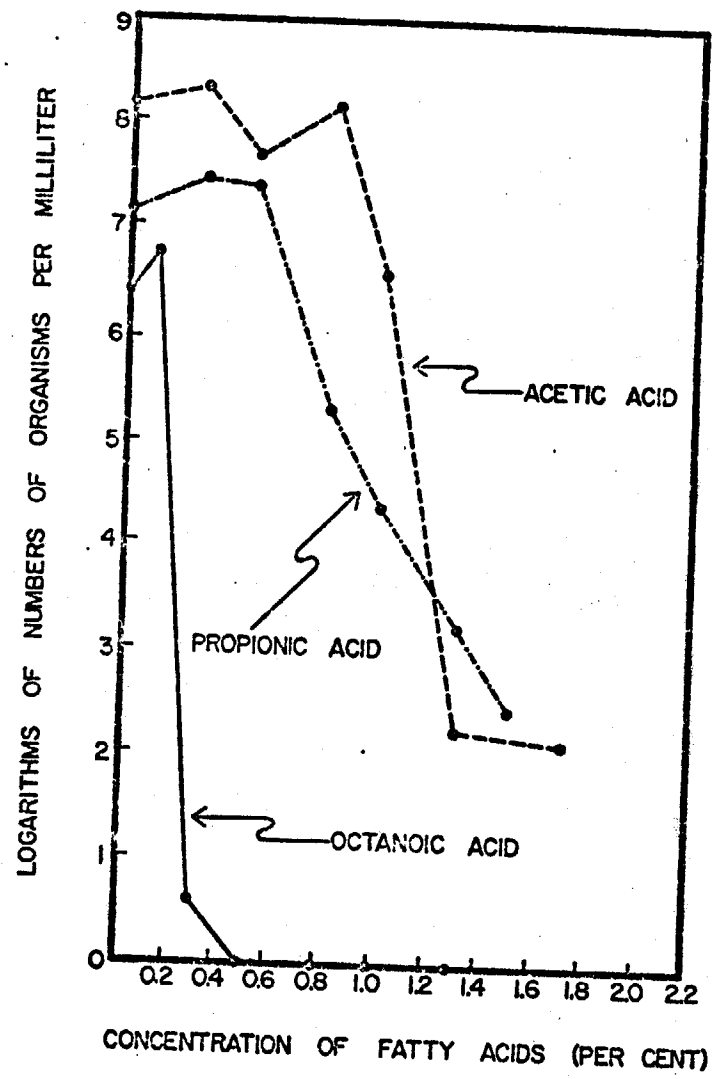

Figure 4. Effect of Acetic, Propionic, and Octanoic Acids on *Shigella sonnei* 6761.

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...sical basis of curves 1 and 2 is ...ward. From the values of ref. ... out that the space constant in ...y state is 1·0 μm, whereas the ...stant for an exponentially rising ...f time constant 0·13 ms is about ...this almost all the tubular net... ...tributes to the low frequency ...y, but only the outer zone to the ... capacity during the foot of the ... potential. The low frequency ...y therefore increases with diam... but C_f is nearly constant for the ...fdiameters between 50 and 150 μm. ...general agreement between theory ...experiment helps to validate the ...of Adrian, Chandler and A. L. H., ...vides strong evidence for attribut... ...the high capacity of twitch fibres to ...transverse tubular system. The estimates of tubular ...surface capacity are in good agreement with those ...ned from impedance measurements by Dr M. ...ander (personal communication).

...N was a Wellcome Trust fellow.

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Effect of Fatty Acids on Contractility and Rhythm of the Heart

SINCE the finding in 1961 that free fatty acids inhibit the myocardial oxygenation of glucose[1], increasing emphasis has been placed on the role of free fatty acids as myocardial fuel[2]. It is now known that free fatty acids inhibit glucose metabolism at several sites[3]. In studies on the human heart *in situ* free fatty acids are the dominant fuel in the fasting state[4]. It has therefore been suggested that free fatty acids are of major importance as a source of myocardial energy.

But circumstantial evidence has grown up to suggest that free fatty acids can in certain circumstances be toxic to the heart. In patients with acute myocardial infarction those with the most free fatty acid in the blood are most liable to develop arrhythmias[5,6]. In dogs with ischaemic lesions of the myocardium, an abrupt increase of the plasma free fatty acids as a result of infusion of heparin and a lipid emulsion is associated with ventricular extrasystoles and ventricular arrhythmias[6]. These effects are on pathological hearts, but we have tested the direct effects of fatty acids on cardiac contractility in normal isolated hearts.

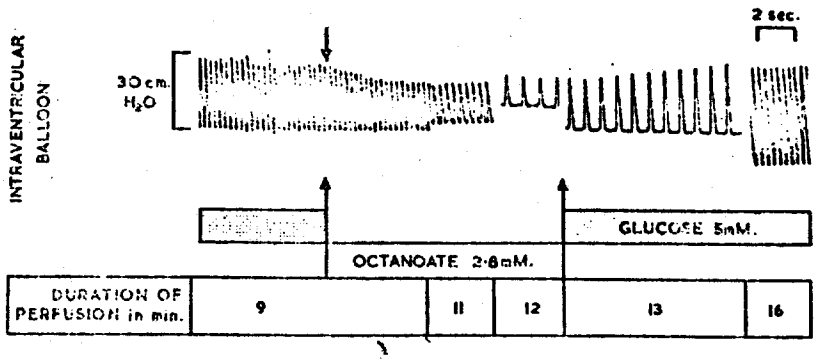

Fig. 1. Effect of octanoate on the contractility of isolated perfused rat heart. When the substrate of the isolated rat heart is changed (marked by arrow) from glucose to 2·8 mM octanoate contraction abruptly decreases and a high-grade atrioventricular conduction defect develops after 3 min. Normal contractions resume 5 min after changing back to a substrate of 5 mM glucose.

Isolated rat hearts were perfused with a Krebs–Henseleit bicarbonate medium, pH 7·4, with 5 mM glucose, or medium chain free fatty acid (octanoate) as substrate. Cardiac contractility was monitored by an intraventricular latex balloon[3]. Octanoate was prepared from octoic acid (British Drug House, Poole, Dorset) which was more than 97·5 per cent pure octoic acid; the rest consisted of other medium chain fatty acids as shown by gas–liquid chromatography. There were no known toxic substances in the octoic acid.

Table 1. EFFECT OF OCTANOATE ON CONTRACTILITY AND HEART RATE OF ISOLATED PERFUSED RAT HEART

Concentration of octanoate (mM)	No. of observations	Peak systolic pressure (per cent)	Heart rate (per cent)
2·800	(4)	59	81
1·400	(4)	83	98
0·700	(2)	62	100
0·350	(1)	69	100
0·175	(2)	70	100

Peak systolic pressure and heart rate are expressed as percentage of values obtained with 5 mM glucose as the only substrate. Mean values with glucose were: peak systolic pressure, 50 cm of H_2O; heart rate, 214 beats/min (fourteen observations in each case).

When the substrate of the isolated heart was abruptly changed from 5 mM glucose to 0·175–2·8 mM octanoate, within 1–2 min the peak systolic pressure fell to 59–83 per cent of the value with glucose as substrate (Table 1). With the highest octanoate concentration (2·8 mM) the effects were: (i) the heart rate decreased; (ii) a 3 : 1 atrioventricular block developed and the peak systolic pressure decreased to one-third after 2–3 min in two experiments (Fig. 1) and (iii) after re-exposure to glucose the rhythm reverted to normal in one experiment (Fig. 1), while in another experiment partial reversal to normal occurred. When the perfusion with 1·4 mM octanoate was prolonged, an irregular rhythm (quadrigeminy) developed after 5 min in one of seven experiments. Prolonged perfusion with 0·7 mM octanoate resulted in no rhythm disturbance.

In other experiments the rate of oxidation of 1·4 mM sodium octanoate-1-^{14}C was 4·2 ± 0·8 (means ± s.e.m., four hearts) μmole of octanoate/g wet weight/15 min, while 0·3 ± 0·06 μmole were recovered in the tissue lipid fraction. When 2·8 mM octanoate was added to 5 mM glucose-U-^{14}C, the rate of oxidation of glucose was reduced to 11 per cent of the control value (four hearts). Although contractility was not measured, no serious arrhythmias developed.

These experiments show that octanoate, a medium chain length fatty acid could decrease the contractility of the isolated perfused rat heart and could cause arrhythmias. No similar changes occurred with albumin-bound long chain free fatty acid (palmitate) in physiological concentrations[3,9] nor were rhythm disturbances noted when both glucose and octanoate were present in the perfusate. The metabolic behaviour of labelled octanoate was similar to that of labelled albumin-bound palmitate.

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Octanoate also inhibited the uptake of glucose and pyruvate by the perfused rat heart[10,11]. When hearts were perfused with both glucose and long chain fatty acid, the formation of triglyceride was increased[12], probably accompanied by a decrease in the tissue concentration of free fatty acid. It is not, however, known whether glucose could encourage the formation of triglyceride from octanoate.

It is attractive to postulate that the accumulation of free intracellular fatty acid is toxic to heart function, for example, by inhibiting oxidative phosphorylation[13]. This could explain (i) the effect of a very high concentration of fatty acid (2·8 mM octanoate) on the heart; (ii) the decreased efficiency of contraction of the isolated atrium when exposed to 2 mM hexanoate as sole substrate[14] and (iii) the effect of very high circulating concentrations of long chain fatty acids on the rhythm of the infarcted heart, because uptake of free fatty acid by the ischaemic segment is proportional to the circulating concentration[15], while oxidation of free fatty acids is probably reduced on account of hypoxia[2]. Furthermore, since completion of this report, high but not low perfusate concentrations of a long-chain fatty acid, linoleate, have also been shown to be toxic to the perfused rat heart[16].

The experiments reported here show that fatty acids in high concentrations can be toxic to the normal heart and can result in decreased contractility and rhythm disturbances.

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Electron Probe Microanalyser Localization of Lead in Kidney Tissue of Poisoned Rats

ATTEMPTS to explain the morphological changes of the kidney induced by lead poisoning have been concerned chiefly with nuclear and cytoplasmic inclusions[1,2]. Cytoplasmic inclusions have been shown to contain Fe (ref. 1), but the composition of nuclear inclusions remains a subject for debate. It is still not clear whether lead is deposited within the kidney and some authors deny a direct action of this element in favour of a mechanism of indirect action through vascular changes[3]. Fortunately, the problem of detection of metallic localizations within the kidney is amenable to direct observations with the electron probe microanalyser, an instrument ideally suited to the purpose, with a detection limit of about 10^{-15} g and a spatial resolution of about 1 μm (ref. 4).

Kidney tissue was taken from Sprague–Dawley rats fed for 72 weeks on 1 per cent lead acetate solution[5]. Conventional paraffin embedded sections were made, mounted on pure silicon wafers, and the paraffin removed by xylene. Such sections, about 20 μm thick, were suitable for pathological purposes. Numerous glomeruli and normal and dilated tubules could be clearly defined under the microscope. In the electron probe (CAMECA), lead was readily found within cortical tubules in granules of differing shape and size. Fig. 1 shows a typical localization within a kidney tubule. Notable concentrations of calcium and phosphorus were found to be associated with the lead. Other elements were probably also present, but the chief constituents of the granules were lead, calcium and phosphorus. The scanning X-ray images (Fig. 1) also showed that calcium and phosphorus, in contrast to lead, appeared weakly throughout the tissue comprising the tubules.

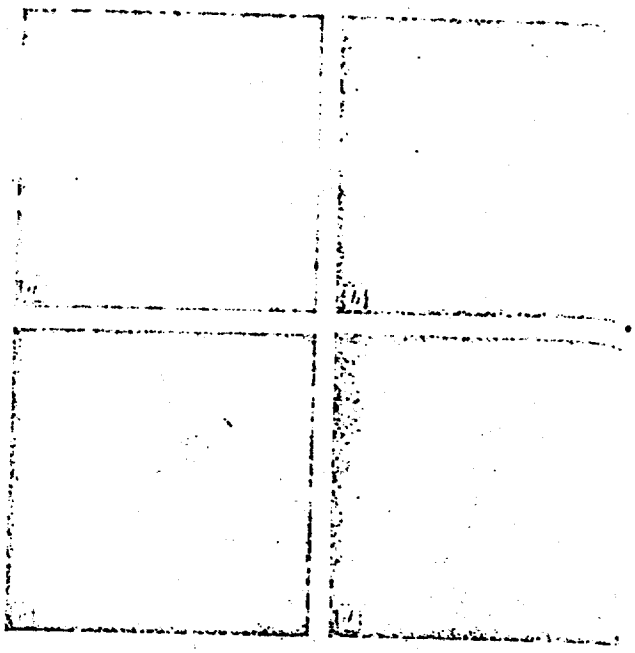

Fig. 1. Electron beam scans of rat kidney tissue, showing location of lead, calcium and phosphorus. Accelerating voltage 35 kV, specimen current 10^{-7} A. Each scan covers the same area of 100 μm × 100 μm. *a*, Specimen current; *b*, lead; *c*, calcium; *d*, phosphorus.

These observations lend support to recent electron microscope work[5] which suggested that lead poisoning is associated with lead–protein complexes. In that study lead was identified by a careful extraction process from many whole kidneys which isolated a quantity of inclusions sufficient for chemical analysis. Our work, in addition to localizing the lead, shows that calcium and phosphorus are co-deposited with the lead in these complexes, a result consistent with the hypothesis of the importance of lead–protein complexes.

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ACETATE AND OCTANOATE UTILIZATION BY LIVER AND ADIPOSE TISSUE OF CASTRATED AND GONADAL HORMONE-TREATED RATS[1]

W. F. Perry and Helen F. Bowen

Abstract

The incorporation of C^{14}-labelled acetate into fatty acids and cholesterol was studied in vitro in castrated and gonadal hormone-treated male and female rats. Measurements were also made on the incorporation of C^{14}-labelled octanoic acid into acetoacetic acid by liver tissue and the incorporation of acetate and octanoate into CO_2.

Castration in the male, but not in the female, was followed by an increased incorporation of acetate into both liver and adipose tissue fatty acids and into liver cholesterol.

Testosterone treatment led to a decreased incorporation into fatty acids by liver tissue in the male, whereas estradiol treatment of the female led to an increase in the incorporation of acetate into fatty acids by both liver and adipose tissue. Acetate incorporation into cholesterol was unaltered by hormone treatment in both sexes. The incorporation of octanoic acid into acetoacetic acid by liver tissue was decreased in the estradiol-treated female rat but was unaltered in the testosterone-treated male rat or by castration in either sex.

Introduction

The metabolism of lipids is modified by a number of hormonal factors, in particular the secretions of the pancreas, pituitary, and adrenal. There is also considerable evidence to implicate the gonadal hormones. In recent years there has been increased interest in the role of gonadal hormones in lipid metabolism with the demonstration in the human of hypercholesterolemic actions of testosterone and the hypocholesterolemic actions of estrogens and the possible application of this to the treatment of atherosclerosis. The findings of various investigators as to the precise effects of the sex hormones on lipid metabolism have frequently differed.

Bates, Zomzely, and Mayer (1) reported that ovariectomy had no effect on acetate incorporation into the liver and carcass fatty acids of intact rats. Both ovariectomy (2) and estrogen treatment (3), however, have been reported to depress hepatic cholesterol synthesis. Fillios (4) claimed that castration in both sexes led to an increase in serum cholesterol and that, on treatment of the castrate animals with their respective sex hormones, estradiol led to a hypercholesterolemia and androgen treatment lowered the serum cholesterol level. Recently Coleman, Chen, and Alfin-Slater (2) have reported experiments which show that, in the rat, castration in either sex does not lead to any alteration in the serum cholesterol level but is followed by a decreased synthesis of cholesterol by liver slices from both male and female castrate. Previously Langdon and Greenberg (5) had concluded that the turnover of cholesterol in the castrate male rat was depressed but that the ability of liver slices from such animals to synthesize cholesterol was unimpaired.

[1]Manuscript received June 26, 1958.
Contribution from the Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Canada.

Cryp. Acid

TABLE I

INCORPORATION OF ACETATE-2-C^{14} INTO FATTY ACIDS, CHOLESTEROL, AND CO_2 BY LIVER AND ADIPOSE TISSUE FROM INTACT AND CASTRATED MALE AND FEMALE RATS

Mean values ± S.E.

	Liver						Adipose tissue			
	Fatty acids		Cholesterol				Fatty acids			
	% inc./g	S.A., c.p.m./15 mg F.A.	% inc./g	γ/g	S.A., c.p.m./mg	CO_2, % inc./g	% inc./mg N	S.A., c.p.m./15 mg F.A.	CO_2, % inc./mg N
Males									
Intact (6)	32.5 ±5.1	9000 ±2000	4.5 ±.57	1760 ±85	2600 ±330	17.2 ±2.0	14.8 ±2.9	13400 ±2200	3.6 ±0.8
Castrate (6)	44.9 ±4.0	15200 ±1300	7.1 ±.83	1680 ±155	4300 ±450	17.3 ±2.1	25.2 ±4.0	31600 ±8900	3.5 ±0.8
P value	.05	<.05	<.05	n.s.	.01	n.s.	<.05	.05	n.s.
Females									
Intact (6)	40.6 ±6.5	14900 ±2500	4.8 ±0.5	1440 ±150	2480 ±460	16.7 ±2.5	18.2 ±3.0	13600 ±3100	2.3 ±0.7
Castrate (6)	38.7 ±5.3	13700 ±1200	4.3 ±0.7	1655 ±170	2100 ±500	15.7 ±2.2	22.1 ±7.8	21000 ±6500	3.2 ±0.5
P value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

It was considered of interest by us to further study the possible effects of gonadal hormones on lipid metabolism by observing the effects of castration and sex hormone treatment on the incorporation of radioactive acetate into fatty acids and cholesterol by slices of liver and adipose tissue and, in addition, the effects on the utilization of radioactive octanoic acid by liver tissue.

Methods

Four-month-old hooded rats of both sexes were used four to six weeks after castration. During this postoperative interval they were pair-fed with intact control animals of the same age. Four hours before killing, both castrates and controls were given 4 g glucose *per os*. Hormone-treated animals were used after 10 days' treatment with testosterone (5 mg daily) in the case of males or estradiol (1 mg daily) in the case of females. Control animals for these groups were injected daily with identical amounts of the sesame oil vehicle alone. The hormone-injected animals and their controls were not given glucose prior to the experiments. The animals were killed by decapitation, the liver was quickly removed, and slices were prepared. Intestinal mesentery was used as a source of adipose tissue. Liver tissue was incubated with a substrate of 2-C^{14}-labelled sodium acetate in studies on hepatic lipogenesis and cholesterogenesis and with 1-C^{14}-labelled octanoate in studies on hepatic fatty acid utilization. Adipose tissue was incubated with 1-C^{14}-labelled acetate as a substrate for fatty acid synthesis by this tissue. The details of the incubation conditions and isolation and measurements of the fatty acids and cholesterol have been described previously (6).

Results

In Table I are shown the results with tissue slices from the castrate animals. Castration in the male led to an increased acetate incorporation into fatty acids with both liver and adipose tissue. There was also increased acetate incorporation into cholesterol by liver slices. The CO_2 production by liver and adipose tissue was unaffected by castration. Castration in the female was followed by no significant difference in the incorporation of acetate into fatty acids, cholesterol, or CO_2 by liver or adipose tissue. As shown in Table II, castration in both sexes was without effect on the ability of the liver to incorporate the carboxyl-labelled carbon of octanoic acid into acetoacetic acid or CO_2.

In Table III are set out the results of gonadal hormone treatment. It will be seen that in the testosterone-treated male rats a marked decrease occurred in the incorporation of acetate into fatty acids by liver tissue. Adipose tissue appeared unaffected. Testosterone treatment did not significantly change acetate incorporation into cholesterol or CO_2. Liver tissue from the estradiol-treated female rats incorporated significantly more acetate into fatty acids than did the slices from control animals. The specific activity of the adipose tissue fatty acids was also significantly increased in the hormone-treated females. However, estradiol treatment had no demonstrable effect

on acetate incorporation into cholesterol or CO_2. In Table IV are shown data on the incorporation of carboxyl-labelled octanoic acid into acetoacetic acid and CO_2 by liver slices of the gonadal hormone-treated rats. It will be seen that testosterone was without effect on the utilization of octanoic acid, whereas in the estradiol-treated females there was a reduction in the incorporation of octanoate carbon into acetoacetic acid by liver tissue compared with liver slices from untreated female rats. In neither sex did hormone treatment alter the amount of octanoate carbon incorporated into CO_2.

TABLE II

INCORPORATION OF OCTANOATE-1-C^{14} INTO ACETOACETIC ACID AND CO_2 BY LIVER SLICES FROM INTACT AND CASTRATED MALE AND FEMALE RATS

Mean values ± S.E.

	Acetoacetic acid			
	% inc./g	γ/g	S.A., c.p.m./mg	CO_2, % inc./g
Males				
Intact (6)	12.8 ±3.2	1590 ±250	28800 ±3500	9.7 ±1.5
Castrate (6)	12.7 ±3.0	1560 ±315	28500 ±2300	10.6 ±2.0
Females				
Intact (6)	10.3 ±5.6	1190 ±170	30000 ±2080	9.3 ±1.3
Castrate (6)	11.7 ±6.0	1410 ±340	28000 ±2530	9.6 ±1.2

Discussion

From the above experiments it would seem that the gonadal hormones can influence lipid metabolism in the rat. The findings have been summarized in Table V. Our results are in agreement with those of Dugal and Saucier (7), who reported an increased incorporation of acetate into cholesterol by liver in the castrated male rat.

Whether this effect on both liver fatty acid and cholesterol is due to a lack of testosterone or to the action of unantagonized estrogens present in the male is not possible to say from the data. If the latter possibility is correct, one could anticipate that estradiol treatment would increase lipogenesis and castration of the female would decrease it. In our experiments an increased incorporation of acetate into fatty acid, accompanied by a decrease in utilization of fatty acid by liver tissue, as reflected by a decrease in octanoate incorporation into acetoacetic acid, was found in the estradiol-treated female but no change was noted in incorporation into cholesterol. This finding is not in agreement with Coleman *et al.* (2), who did find a reduction in hepatic cholesterogenesis in vitro in the castrated

TABLE III

INCORPORATION OF ACETATE-2-C^{14} INTO FATTY ACIDS, CHOLESTEROL, AND CO_2 BY LIVER AND ADIPOSE TISSUE FROM INTACT AND TESTOSTERONE-TREATED MALE AND ESTRADIOL-TREATED FEMALE RATS

Mean values ± S.E.

	Liver						Adipose tissue			
	Fatty acids		Cholesterol				Fatty acids			
	% inc./g	S.A.	% inc./g	γ/g	S.A.	CO_2, % inc./g	% inc./mg N	S.A.	% inc./mg N	CO_2, % inc./mg N
Males										
Intact (6)	23.0 ±2.7	6500 ±1070	1.8 ±4.0	1430 ±120	7800 ±2800	13.3 ±3.5	4.5 ±2.1	4450 ±1250	2.1 ±0.9	
Testosterone (6)	11.5 ±2.6	3140 ±690	1.7 ±0.6	1430 ±165	6500 ±1100	11.6 ±2.7	4.0 ±2.3	3410 ±1300	3.1 ±0.4	
P value	<.01	<.05	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
Females										
Intact (4)	15.6 ±1.4	4810 ±1110	2.9 ±1.3	1420 ±100	13800 ±3200	11.9 ±2.8	2.1 ±1.7	2010 ±310	2.1 ±0.8	
Estradiol (4)	27.8 ±1.8	8250 ±975	2.3 ±1.5	1390 ±85	11000 ±2100	12.4 ±3.1	2.5 ±1.3	3700 ±800	1.3 ±0.6	
P value	<.05	.05	n.s.	n.s.	n.s.	n.s.	n.s.	<.05	n.s.	

female rat. However, the finding of no change in acetate incorporation into liver fatty acids in the female after castration is in agreement with the report of Bates *et al.* (1) to the effect that ovariectomy was without effect on acetate incorporation into liver fatty acid.

TABLE IV

INCORPORATION OF OCTANOATE-1-C^{14} INTO ACETOACETIC ACID AND CO_2 BY LIVER SLICES FROM INTACT AND TESTOSTERONE-TREATED MALE AND ESTRADIOL-TREATED FEMALE RATS

Mean values ± S.E.

	Acetoacetic acid			
	% inc./g	γ/g	S.A.	CO_2, % inc./g
Males				
Intact (6)	16.3 ±1.7	1320 ±200	33400 ±3000	11.1 ±2.0
Testosterone (6)	15.2 ±2.3	1350 ±185	29000 ±2100	12.0 ±2.5
P value	n.s.	n.s.	n.s.	n.s.
Females				
Intact (4)	19.6 ±3.2	1720 ±285	28100 ±3210	11.9 ±3.2
Estradiol (4)	11.6 ±2.1	1530 ±285	19400 ±2530	12.4 ±1.3
P value	.05	n.s.	.05	n.s.

TABLE V

EFFECT OF CASTRATION AND GONADAL HORMONES ON ACETATE AND OCTANOATE UTILIZATION

	Lipogenesis	Cholesterogenesis	Octanoate utilization
Male : castrate	Increased	Increased	Unchanged
Female : castrate	Unchanged	Unchanged	Unchanged
Male + testosterone	Decreased	Unchanged	Unchanged
Female + estradiol	Increased	Unchanged	Decreased

Acknowledgment

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Diabetes 18(2): 96-100 (1969)

Insulin and Ketone Responses to Ingestion of Medium and Long-chain Triglycerides in Man

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with technical assistance of Eva deLooze

New York

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SUMMARY

The effects of medium chain triglyceride (MCT), corn oil, and water on serum glucose, ketones, and immunoreactive insulin (IRI) were compared. Fourteen normal subjects ingested 1 gm. per kg. of MCT (composed principally of C8 and C10 fatty acids) and on another occasion an equal volume of water. After water ingestion, glucose, ketones, and IRI did not change appreciably up to five hours. After MCT, a significant rise in serum ketones and IRI was associated with a slight fall in serum glucose. Fourteen normal subjects ingested 1 gm. per kg. corn oil, and on another occasion an equal volume of water. Serum glucose, ketone and IRI concentrations again did not change up to five hours after ingestion of water. After corn oil, no significant rise in ketones occurred. There was a small rise in IRI and a slight fall in glucose. In the eight subjects in whom MCT, corn oil and water were compared a certain degree of insulin secretory response was obtained after MCT and corn oil but not after water ingestion. However, the IRI response after MCT was highest. The rise in IRI without a corresponding change in serum ketones after ingestion of the long chain triglyceride might be attributed to a gastrointestinal β-cytotropic effect. The mechanism of IRI stimulation by MCT also has been related to MCT-induced hyperketonemia and possibly to a direct effect on insulin secretion of small amounts of octanoate in peripheral blood. DIABETES *18*:96-100, February, 1969.

The hypoglycemia induced by administration of acetoacetate and other ketones has been ascribed to an insulin stimulatory effect.[1] The degree of hyperketonemia at which this stimulatory effect occurs has not been clarified. The ingestion of medium chain triglycerides can induce a modest hyperketonemia in man.[2,3] The purpose of the present study was to ascertain the insulin secretory response to medium chain triglyceride-induced hyperketonemia in normal human subjects. Also, control experiments were conducted to estimate the insulin secretory response of subjects to the ingestion of a long chain triglyceride or simply water.

MATERIALS AND METHODS

Initially, fourteen normal subjects (ten men and four women) were studied on two separate mornings at least one week apart. All subjects were lean, healthy volunteers, eighteen to thirty-five years of age. Following overnight fast, each individual ingested either 1 gm. per kg. body weight of medium chain triglyceride (MCT) or an equivalent volume of water. The fatty acid composition of the MCT preparation was approximately 80 per cent octanoate (C8) and 17 per cent decanoate (C10) with traces of laurate (C12) and hexanoate (C6).[4]

Additional studies were made by feeding volunteers 1 gm. per kg. body weight of long chain triglyceride (corn oil). Eight of the original volunteers and six new ones were available for this purpose. The MCT or the corn oil was emulsified by ultrasonication in an equal amount of water containing 0.5 ml. of Tween 80. The same amount of Tween 80 was added to the water control "meal." Venous blood samples were obtained before and for five hours after the ingestion of either the fat or the water. After the blood was permitted to clot on ice for three hours, the serum was separated and immediately frozen until use. The serum samples were analyzed for glucose,[5,6] total ketones,[7,8] and immunoreactive insulin.[9,10] The immunoreactive insulin (IRI) was measured against a human insulin standard.* Duplicate values of the per cent activity of the bound insulin precipitates had to agree within 3 per cent to be considered valid.

From the Department of Medicine, St. Luke's Hospital Center and the Institute of Nutrition Sciences, Columbia University, New York, New York.

*Kindly supplied by Dr. Mary Root, Lilly Laboratories, Indianapolis, Indiana.

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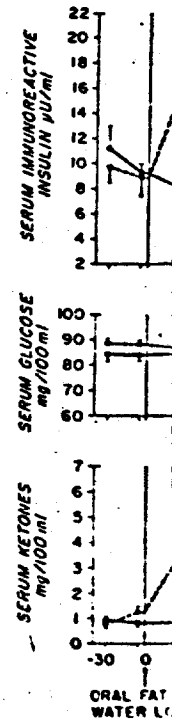

FIG. 1. Mean se
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RESULTS

The results obtained in the fourteen subjects who took MCT or water are shown in figure 1. In presentation of the results, all values represent means unless otherwise specified, those described as "baseline" represent the average of the pre-ingestion means measured at —30 and —5 min. The baseline postabsorptive serum ketone levels in the fourteen subjects studied were 0.77 ± 0.26 (S.E.) mg. per 100 ml. After water ingestion serum ketones remained at a level below 1.3 mg. per 100 ml. After MCT serum ketones rose to a peak, however, of 6.7 mg. per 100 ml. at two hours, and then were maintained at a level above 6.0 mg. per 100 ml. for five hours. Serum insulin concentrations ranged from 6.6 to 11. 2 μU. per ml. throughout the water ingestion period, the average of these means being 8.2 μU. per ml. After MCT insulin rose from a baseline level of 9.3 μU. per ml. to values of 17.7 μU. per ml. at thirty minutes ($p < .005$), 15.3 μU. per ml. at one hour ($p < .005$) and 15.4 μU. per ml. at two hours ($p < .05$). Values at four and five hours were elevated above the baseline value, but the differences were not significant. Serum glucose remained constant at about 85 mg. per 100 ml. before and after water ingestion. After MCT there was a slight fall in serum glucose values varying between 6 and 8 mg. per 100 ml. which, after one hour, were significantly lower ($p < .005$) than the preingestion values.

The results in the fourteen volunteers who ingested corn oil and then water are shown in figure 2. Serum ketones did not change significantly after water ingestion, remaining at a level below 1.2 mg. per 100 ml. After corn oil, ketones rose very slightly with the highest level of 2.0 mg. per 100 ml. recorded at five hours. Serum insulin concentrations after water did not rise but actually fell slightly below the baseline level of 10.4 μU. per ml. After corn oil, insulin rose from a baseline concentration of 10.9 μU. per ml. to values of 12.7 μU. per ml. at thirty minutes, 13.4 μU. per ml. at one hour, 13.8 μU. per ml. at two hours, diminishing again to 11.9 μU. per ml. at four hours and 10.2 μU. per ml. at five hours. With the exception of the two-hour concentration ($p < .05$), these values did not differ significantly from the baseline level. Serum glucose did not change during water ingestion, while after corn oil there was a slight fall in glucose which, at sixty minutes, was lower than the pre-ingestion values ($p < .05$).

A comparison of results obtained after water, corn oil

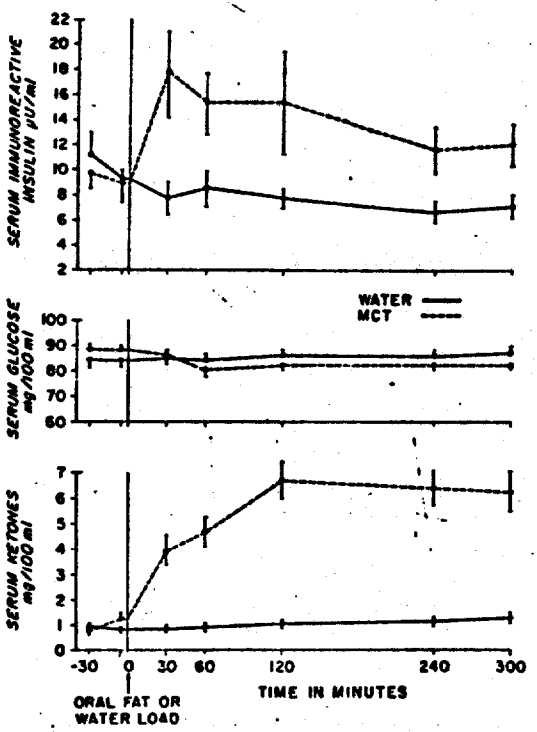

FIG. 1. Mean serum immunoreactive insulin, glucose, and ketone responses (± S.E.) to ingested water and MCT in fourteen normal subjects.

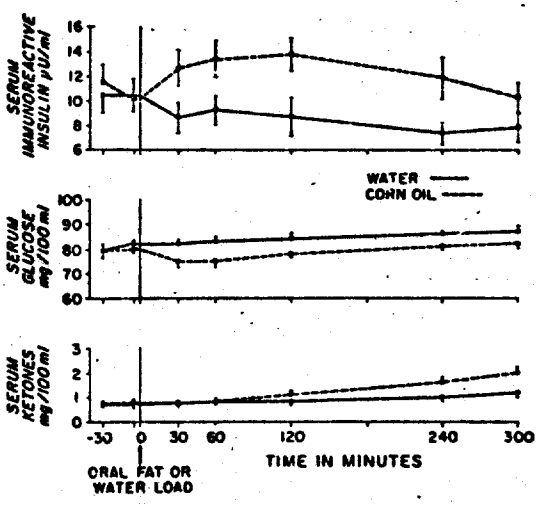

FIG. 2. Mean serum immunoreactive insulin, glucose, and ketone responses (± S.E.) to ingested water and corn oil in fourteen normal subjects.

and MCT in the eight subjects who underwent all three studies is shown in figure 3 and table 1. When the IRI responses to MCT were compared with those after water, there was a significantly ($p < 0.025$) higher IRI response throughout the period following ingestion of MCT. In contrast, when IRI responses to corn oil were compared with those obtained after water ingestion, significantly higher IRI responses were detected only at thirty and 120 minutes. When MCT was compared with corn oil, significantly higher IRI responses were obtained with MCT at thirty minutes.

DISCUSSION

MCT has been shown previously to induce modest hyperketonemia in man.[2,3,11] This phenomenon has been related to the perfusion of the liver by medium chain fatty acids which travel in the portal vein after digestion and absorption of the fatty acids and monoglyceride derivatives of the parent triglyceride.

The hypoglycemic action of ketone bodies infused into animals has been reported by a number of investigators.[12,13] The hypoglycemia induced by ketone administration in portacaval shunted dogs by Mebane and Madison[14] was ascribed entirely to a reduction in hepatic glucose output. Subsequently, Madison et al.[1]

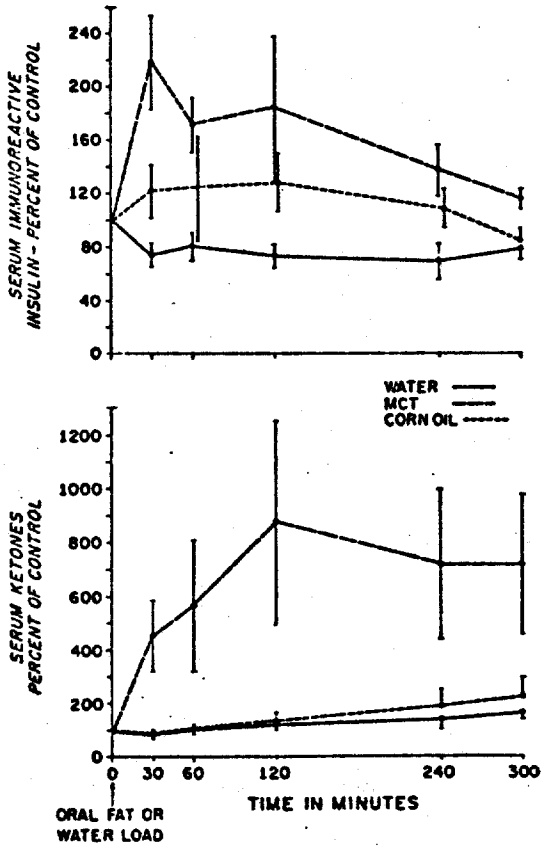

FIG. 3. Mean serum immunoreactive insulin and ketone responses (per cent of control ± S.E.) to water, corn oil, and MCT ingestion in eight normal subjects.

TABLE 1

Mean serum immunoreactive insulin (IRI), glucose, and ketone responses (± S.E.) to water, corn oil, and MCT ingestion in eight normal subjects. Values in parentheses denote per cent of control, with 100 per cent being the average of the two pre-ingestion values at —30 and —5 minutes.

Time in minutes		−30	−5	+30	+60	+120	+240	+300
	IRI	10.5±2.6	8.7±1.8	7.1±1.6 (74)	7.8±1.5 (81)	7.0±1.2 (73)	6.6±1.2 (69)	7.5±1.7 (78)
Water	Glucose	83±2.8	85±2.3	84±3.1 (100)	85±2.9 (101)	86±3.8 (102)	88±2.8 (105)	89±3.4 (106)
	Ketones	0.9±0.2	0.7±0.2	0.7±0.1 (88)	0.8±0.2 (103)	0.9±0.2 (120)	1.1±0.2 (136)	1.3±0.3 (161)
	IRI	10.9±2.3	10.1±1.7	12.8±2.5 (122)	13.0±2.5 (124)	13.3±1.5 (127)	11.4±2.7 (109)	8.9±1.7 (85)
Corn oil	Glucose	82±3.6	82±2.3	77±3.9 (94)	76±3.4 (93)	79±2.5 (96)	82±2.6 (100)	83±2.8 (101)
	Ketones	0.8±0.2	0.9±0.3	0.8±0.2 (87)	0.9±0.2 (105)	1.1±0.2 (129)	1.6±0.2 (185)	1.9±0.2 (221)
	IRI	10.7±2.1	8.4±1.6	20.8±6.5 (218)	16.4±4.5 (172)	17.6±7.2 (184)	13.1±2.8 (137)	11.1±2.2 (116)
MCT	Glucose	87±3.6	87±1.3	85±2.0 (98)	78±2.2 (90)	83±2.5 (95)	81±2.4 (93)	82±1.6 (94)
	Ketones	0.8±0.2	1.0±0.2	4.1±0.7 (450)	5.1±0.9 (561)	7.9±0.9 (870)	6.6±0.9 (712)	6.5±1.1 (718)

demonstrated enhanced insulin levels in pancreatic venous blood after ketone infusions, but these workers could not obtain a similar hypoglycemia in depancreatized dogs. The suggestion was made that the modestly increased insulin levels induced by hyperketonemia dampen further ketone production by regulating free fatty acid release from adipose tissue thereby preventing severe ketoacidosis during starvation. Since no direct ketone measurements were made by Madison et al., the degree of ketonemia at which an insulin stimulatory effect would occur was left undefined, albeit these authors estimated that an insulin secretory effect occurred at levels of 3.5 mMoles per liter (approximately 21 mg. per 100 ml. of acetone equivalent)

In the present experiments, the modest hyperketonemia induced by MCT was associated with a small rise in circulating IRI. Although serum IRI concentrations of 18 μU. per ml. would be considered within the normal postabsorptive range, the changes that occurred after MCT were not evident after water. The change in serum IRI in response to MCT may be related in part to the hyperketonemia. This interpretation is consistent with the studies in dogs by Madison et al.[1] However, Fajans et al.[15] attained plasma ketone values of 25 mg. per 100 ml. after infusing acetoacetate into normal volunteers and were unable to detect a rise in plasma IRI. Moreover, infusion of β-hydroxybutyrate into man has not been found by others[16,17] to enhance insulin secretion.

The possibility that octanoate is capable of directly stimulating release of insulin by the beta cells of the pancreas should be considered. Sanbar et al.[18] infused octanoate and found a slight increase in peripheral plasma IRI in three of the six dogs studied. Other studies of infusion of octanoate in dogs have not shown enhanced insulin secretion.[19] In any event, these studies involve direct introduction of octanoate into the systemic circulation. Under the conditions of the present study, MCT ingestion results in transport of octanoate and decanoate into the portal vein and the liver where these acids are extensively metabolized.[20] Thus, the ingestion of MCT is not associated with an appreciable rise of MCT-derived acids in the systemic circulation.[21] Whether the small rise in arterial blood octanoate that might occur after MCT ingestion could directly stimulate insulin secretion cannot be determined from the present study.

Since serum ketones exhibited virtually no rise after corn oil, it is unlikely that they played a role in the small rise in circulating IRI observed. However, the presence of ingested fat in the gastrointestinal tract may result in the release of certain intestinal hormones. Some of these hormones have been shown by Unger et al.[22] to stimulate insulin secretion. Thus, it is possible that the enhancement of insulin secretion in response to corn oil ingestion may be ascribed to a direct effect of the fat load on the gastrointestinal tract with release of β-cytotropic hormones. However, direct evidence for this possibility is not provided by the present study.

When the IRI responses were compared after MCT, corn oil, and water in the same eight subjects, it was evident that MCT induced the highest insulin response. Thus, the mechanism of IRI stimulation by MCT must be related to an effect other than a possible fat-mediated gastrointestinal hormonal release. Whether the higher degree of insulin response to MCT ingestion is induced by the hyperketonemia or the small amount of octanoate that may have escaped hepatic metabolism cannot be determined from the present study.

ACKNOWLEDGMENT

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Dr. Pi-Sunyer is a Fellow of the New York Heart Association and Dr. Hashim is Career Development Investigator, National Heart Institute.

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Body Odor and Metabolic Defects

...N-Butyric/N-hexanoic acidemia is another rare condition in which the exhaled air, tissues, and body fluids have the strong odor of sweaty feet. This condition has so far been observed in two families, involving four and possibly five infants (J. B. Sidbury, Jr., W. R. Harlan, and B. Wittels, Am. J. Dis. Child. *104*:531, 1962; Sidbury, E. K. Smith, and Harlan, J. Pediat. 70:8, 1967). This abnormality of short chain fatty acid metabolism, in which the odor is caused by butyric and hexanoic acids, is probably an autosomal recessive disorder and is due to a deficiency of the acyl dehydrogenase specific for the metabolism of four and six chain fatty acids. Feeding difficulties, lethargy, weakness, and odor developed in these children in the first days of life and were followed by severe acidosis, dehydration, and convulsions reminiscent of tetany. Death ensued in the first month from bone marrow depression with pancytopenia, a bleeding diathesis, and gram negative sepsis.

Other rare disorders which must be considered when an infant has a peculiar odor include the oasthouse syndrome, hypermethioninemia, and methionine malabsorption.

The child with oasthouse syndrome described by A. J. Smith and L. B. Strang (Arch. Dis. Child. 33:109, 1958), had a distinctive unpleasant pungent smell reminiscent of a brewery or oasthouse. The clinical pattern was like that of phenylpyruvic oligophrenia, but differed in unresponsiveness to stimuli, muscular hypotonicity, recurrent episodes of fever, tachypnea, and edema. White hair grew at nine months and death occurred at ten months of age. A double metabolic defect existed, since phenylketonuria was also present. The smell of the urine differed from the mousy odor of phenylacetic acid, which is commonly excreted in phenylketonuria, and was thought due to an oxidation or polymerization product of alpha-hydroxybutyric acid....

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THROMBOTIC, ATHEROSCLEROTIC AND LIPEMIC EFFECTS OF DIETARY FATS IN THE RAT

SERGE RENAUD, V.M.D., PH.D.*

A few years ago, Pickering[1] noted, "The great weakness of the lipid hypothesis is that it does not account for the thrombus." He did not consider as satisfying to a clinician that alterations in lipid metabolism could also predispose to thrombosis. Nevertheless, numerous studies have indicated a relationship between thrombosis and hyperlipemia,[2-4] as well as the importance of lipids in blood clotting[5]; in addition, there is a strong possibility that the long-chain saturated fatty acids of the dietary fat are responsible for thrombosis[6] being by far the most effective fatty acids in aggregating blood platelets *in vitro*.[7, 8]

In recent experiments in rats, we devised an experimental model[9] that results in a constant and extremely elevated incidence of easily observed large occlusive thromboses, provided the animals are fed certain high-fat diets for 6 to 10 weeks.[10] Thrombosis is then initiated by the intravenous injection of a Gram-negative bacteria endotoxin, resulting in multiple, red hepatic infarcts, and a simple quantitative evaluation of the phenomenon can be achieved merely by weighing the lesions, as will be described in this paper.

By adding fatty acids to the basic high-fat diet, we were able to show[11-13] that, under these conditions, as *in vitro*, the long-chain fatty acids were once again the most thrombogenic and that stearic acid appeared to be the most active among them. However, the very-long-chain fatty acids, such as behenic acids, were not thrombogenic, probably because of very poor digestibility.[12, 13]

In these studies in rats, as in man,[14, 15] palmitic acid was the most hypercholesterolemic of the fatty acids used, whereas stearic acid or short-chain fatty acids such as caprylic acid were much less hyperlipemic. As was also shown in man,[16] only linoleic and linolenic acids, but not oleic acid, were hypolipemic.

Other investigators working on the relationship between the human diet and its lipemic effects[15] recently suggested that people wishing to avoid coronary disease should substitute cacao butter for butter in the diet, because the former is richer in stearic acid, a long-chain saturated fatty acid that is much less hypercholesterolemic than palmitic, myristic and lauric acids. Judging from the results of work with blood platelets *in vitro*, such recommendations could be disastrous for coronary patients.

Inasmuch as our previous findings with dietary fatty acids seemed to have established the essential link between lipemia in man and the work with

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* Scholar of the Medical Research Council.

blood platelets and coagulation *in vitro*,[12, 13] it was deemed necessary, because of the obvious implications, to make a detailed study under our experimental conditions of most of the common fats utilized in human nutrition. Our aims in these experiments were to verify: (1) whether the thrombogenic, atherogenic and lipemic effects of a dietary fat could be predicted from its composition in fatty acids; (2) to what extent the degree of lipemia as expressed by the serum cholesterol or triglyceride level could be a reliable indication of the thrombogenic or atherogenic capacity of a fat.

In addition, since we had not previously studied myristic and lauric acids, and a knowledge of their effect under our experimental conditions was essential for the present work on dietary fats, these fatty acids were studied here in a small experiment in comparison with caprylic, palmitic and stearic acids.

MATERIAL AND METHODS

Male Holtzman rats with an initial body weight of 150 to 170 gm were utilized. For each experiment, the exact number of animals actually utilized is shown in the corresponding table. The results of experiment 2 (table 2) and experiment 3 (table 4) represent the pooled data of several sets of experiments, with 6 animals per group in each set. These data were pooled because they were practically identical and resulted from experiments performed at approximately the same time and under comparable conditions. The rats were housed in a constant temperature environment and given, *ad libitum*, tap water and the hyperlipemic diet described in detail elsewhere.[10, 11] In the tables are listed the ingredients composing the diets utilized in the corresponding experiment. The casein, cellulose (Alphacel), cholesterol, salt mixture (Wesson), sodium cholate, sucrose and vitamin mixture (vitamin diet fortification mixture) were obtained mixed in the right proportion by the supplier. The fatty acids utilized in experiment 1 (table 1) were also purchased from the same source (Nutritional Biochemicals Corporation). The animal fats utilized were ground and then melted in a steam bath; a sample of each of these tallows was analyzed by gas-liquid chromatography and the results are reported in table 3. Except for the coconut oil (Matheson, Coleman and Bell) and cacao butter, kindly supplied by Fry-Cadbury Ltd., all the fats and oils were commercially available and were therefore purchased from local stores. When the diets were prepared, isocaloric amounts of the fat (melted when necessary) were carefully mixed with the basic ingredients and then kept under refrigeration for not more than 2 weeks.

At the end of the feeding periods and following 17 hr of fasting, approximately 3 ml of blood were removed in siliconized syringes, equipped with 20-gauge needles, from the jugular vein of the rats, under light ether anesthesia, as reported in detail elsewhere.[11] To initiate thrombosis in experiments 1, 2 and 3 (tables 1, 3 and 4), a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide (0.3 mg per kg) in saline was injected by the same needle, after the last withdrawal of blood.

In each rat of the first three experiments, the plasma clotting time after recalcification was performed in duplicate, in plastic tubes (polycarbonate No. 2804, International Equipment Company, Boston), according to the technique already reported.[11] Triglycerides and total cholesterol analysis were performed on individual sera. The triglycerides were determined by the Van Handel method.[17] In experiment 3 (table 4), Florisil (60 to 100 mesh, Fisher Scientific Company) was used, instead of zeolite as suggested by other investigators[18], but chloroform was still kept as the extracting fluid. Only 0.3 ml of serum was utilized for this determination. The total serum cholesterol was determined by a manual adaptation of the Technicon Auto-Analyzer automated ferric chloride technique,[19] which yielded remarkably reproducible results. In brief, 0.2 ml of serum was added to 4.8 ml of isopropanol, carefully agitated and, after standing for 15 min., centrifuged for 5 min. To 0.5 ml of the supernatant were first added 4 ml of a 0.05 per cent ferric chloride solution in 95 per cent acetic acid and then 3 ml of concentrated sulfuric acid. After 5 min. of centrifugation, the reading was made at 550 mμ against a blank.

On 6 rats in each group of experiment 3 (table 4), no serum determinations were carried out; instead, heparinized plasma was collected on ice to determine the fibrinogen, according to the method of Goodwin[20] utilizing only 0.5 ml of plasma, and the unesterified fatty acids by the technique of Itaya.[21]

In the thrombosis experiments, the time of death was recorded and the animals were autopsied. Survivals were killed at 24 hr. with chloroform. Except in experiment 2 (table 2), in which the lesions were graded according to a scale of 0 to 3 as described elsewhere,[13] a quantitative evaluation of the thrombotic phenomenon could be obtained by weighing the liver before and after removing the red infarcts, as shown in figure 1. The severity of the thrombotic phenomenon was then expressed as the percentage in weight of infarcted liver.

In the atherosclerosis experiment (table 5), the animals were killed with chloroform after the last blood removal and the heart and the aorta were dissected as far as the common iliac arteries. The severity of the aortic fatty streaks was then graded according to a scale of 0 to 3 as already described,[13] under binocular examination of the fresh aorta, opened longitudinally. Macroscopic readings were verified by routine histologic examination of the heart and aorta.

RESULTS

Experiment 1 (table 1). Depending on the fatty acid added to the already high fat diet, the results obtained differed markedly. Palmitic acid gave rise to by far the highest values for cholesterol and triglycerides; the next most hyperlipemic fatty acids were caprylic and myristic acids, followed by stearic acid; lauric acid was the least hyperlipemic. In group 5 fed stearic acid, the plasma clotting time was the shortest and the severity of thrombosis the highest; this was followed by the results for group 4 in which the clotting time was significantly longer and the thrombotic lesions were less severe, although not

TABLE 1

Experiment 1: thrombogenicity of various fatty acids

Results = mean ± S.E. Duration of the experiment, 6 weeks. Diet composition in addition to the fatty acid (5 per cent) listed below: butter 32, casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2, water 1 (weight per cent).

Group and Fatty Acid	No. of Carbon Atoms	No. of Animals	Cholesterol	Triglycerides	Plasma Clotting Time	Thrombosis (Severity)	Survival
			mg/100 ml		*sec.*		*hr.*
1. Caprylic...	8	7	720 ± 172	156 ± 24	146 ± 8	2.0 ± 1.1	9.3 ± 2.3
2. Lauric.....	12	6	401 ± 33	111 ± 20	198 ± 17	2.3 ± 1.6	12.7 ± 3.4
3. Myristic...	14	7	738 ± 37	154 ± 16	181 ± 9	0	7.4 ± 2.8
4. Palmitic...	16	7	1153 ± 135	258 ± 39	143 ± 8	8.2 ± 3.6	3.3 ± 1.5
5. Stearic.....	18	7	528 ± 74	130 ± 15	121 ± 3	14.5 ± 6.3	11.4 ± 2.5

significantly so. The thromboses in the groups fed caprylic, lauric and myristic acids were much less severe, but only in groups 2 and 3 was the clotting time prolonged. The survival time was shortest in group 4, where the lipemia was seen, and longest in groups 2 and 5, where the lowest cholesterol and triglyceride levels were observed.

Experiment 2 (table 2). From experiment 1, it could be concluded that stearic acid was the most thrombogenic of the fatty acids studied. Therefore, if these results were to have practical applications for dietary fats, the fats richest in stearic acid should be the most thrombogenic. Table 3 shows the composition of various common fats differing in the type of fatty acids they contain, particularly in the quantity of stearic acid. From this table it can be seen that cacao butter contains 35 per cent stearic acid, butter 14.6 per cent, lard 7.8 per cent and corn oil 3.4 per cent.

As is seen in table 2, after only 8 weeks of dietary feeding, the severity of thrombosis of the butter-fed animals was 0.6 and the mortality rate was 30 per cent. The animals fed cacao butter presented a shorter clotting time, a severity of thrombosis of 2.1, and a 100 per cent mortality rate. Here, however, with cacao butter as the only fat, cholesterol and triglycerides were higher than with butter. After 10 weeks of dietary feeding, the severity of thrombosis in group 3 fed butter was 1.9. The serum cholesterol and triglycerides were no higher than after 8 weeks, but the clotting time was considerably decreased from 185 to 139 seconds. In group 4 fed lard, the severity of thrombosis was only 0.8; the cholesterol and triglycerides were lower and the clotting time longer than in group 3. The mere fact of reducing the butter content to 32 per cent and substituting 6 per cent of corn oil (group 5) markedly prolonged the clotting time and reduced the lipemia; the severity of thrombosis was only 0.4. When corn oil was the only fat in the diet (group 6), the triglycerides, but not the cholesterol, showed a further decrease as compared with group 5; the clotting time was prolonged and the severity of thrombosis decreased to 0.1.

TABLE 2

Experiment 2: thrombogenicity of various fats

Results = mean ± S.E. Diet composition in addition to the ingredients listed below: casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Group and Duration	No. of Animals	Cholesterol	Triglyceride	Plasma Clotting Time	Thrombosis (Severity, 0-3)	Mortality	Survival
		mg/100 ml		*sec.*		%	*hr.*
8 weeks							
1. Cacao butter 32 + water 6....	23	830 ± 23	148 ± 6	155 ± 7	2.1	100	4.2 ± 0.7
2. Butter 38.....	12	585 ± 80	135 ± 4	185 ± 12	0.6	30	18.1 ± 2.3
10 weeks							
3. Butter.....	33	487 ± 45	134 ± 12	139 ± 4	1.9	59	13.9 ± 1.6
4. Lard 32 + water 6.....	12	459 ± 25	115 ± 6	148 ± 8	0.8	58	14.2 ± 2.7
5. Butter 32 + corn oil 6.....	24	340 ± 21	94 ± 9	164 ± 11	0.4	46	16.7 ± 1.8
6. Corn oil 32 ± water 6.....	12	366 ± 1	75 ± 4	185 ± 7	0.1	16	23.0 ± 0.7

TABLE 3

Composition of certain fats in the commonest fatty acids

Selected values adapted from, "Fatty acids in animal and plant products," by V. R. Goddard and L. Goodall, Human Nutrition Research Division, United States Department of Agriculture, 1959.

Fatty Acids (Carbon Atoms)	Coconut Oil	Butter*	Cacao Butter	Bovine Tallow*	Lard	Chicken Tallow*	Horse Tallow*	Olive Oil	Arachis Oil	Corn Oil
Lauric (12).....	45.4	1.1								
Myristic (14).....	18.0	9.1		3.3		1.0	5.6			0.1
Palmitic (16).....	10.5	28.6	24.4	24.9	32.2	24.6	29.6	9.2	8.2	8.3
Stearic (18).....	2.3	14.6	35.0	16.9	7.8	6.9	4.9	2.0	6.2	3.4
Arachidic (20).....	0.4							0.2	4.0	0.4
Lignoceric (24).....									3.9	0.2
Palmitoleic (16:2H)...	1.3	2.7		5.3		6.5	6.0			1.2
Oleic (18:2H).....	7.1	33.0	28.2	46.3	48.0	45.3	31.3	83.1	52.9	30.0
Linoleic (18:4H).....	0.4	2.4	2.4	3.3	11.0	15.7	8.2	3.9	24.7	54.1
Linolenic (18:6H)....		1.4			0.6		15.0			0.6
Iodine index.....	7.8	33.4	37.0	48.3	64.0	79.3	82.7	84.4	90.1	126.7

* Determined in the laboratory of Professor J. M. Demers, Biological Sciences, University of Montreal.

Experiment 3 (table 4). In this study, the results of four different experiments that gave almost identical results were pooled. In table 4 are reported all the data obtained in these experiments; the different fats are listed in order of their decreasing thrombogenic capacity. Although all of the determinations were performed under rigid control conditions by the most reliable methods found to be compatible with the handling of a large number of samples by well-trained technicians, none of the parameters examined seem to have any connection with the severity of thrombosis, and only the survival time appears to be related to the triglyceride level. Figure 2 shows a correlation ($r = -0.81$)

TABLE 4

Experiment 3: thrombogenicity of various fats

Results = Mean ± S.E. Total duration of the experiment = 9 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water): casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Group and Fat	Iodine Index	No. of Animals	Cholesterol	Triglycerides	Unesterified Fatty Acids	Fibrinogen	Plasma Clotting Time	Thrombosis (Severity)	Survival
			mg/100 ml		*μmoles/l*	*mg/100 ml*	*sec.*		*hr.*
1. Butter	33.4	50	693 ± 53	192 ± 22	650 ± 20	380 ± 20	130 ± 2	16.2 ± 2.1	6.8 ± 1.0
2. Arachis oil	91.8	12	720 ± 50	78 ± 9			160 ± 7	9.4 ± 2.5	18.3 ± 1.7
3. Coconut oil	7.8	25	685 ± 37	125 ± 6	500 ± 50	340 ± 10	161 ± 7	4.8 ± 1.7	10.8 ± 1.8
4. Horse tallow	52.7	25	231 ± 20	45 ± 4	420 ± 10	420 ± 10	131 ± 6	4.8 ± 1.3	16.6 ± 1.7
5. Chicken tallow	79.3	28	459 ± 40	80 ± 8	460 ± 20	390 ± 10	141 ± 4	3.7 ± 0.9	16.7 ± 1.4
6. Olive oil	81.4	23	1022 ± 101	132 ± 13	370 ± 40	400 ± 20	144 ± 4	0.9 ± 0.4	11.1 ± 2.0
7. Bovine tallow	48.3	28	1016 ± 100	189 ± 19	460 ± 20	450 ± 40	165 ± 6	0.8 ± 0.4	9.3 ± 1.4

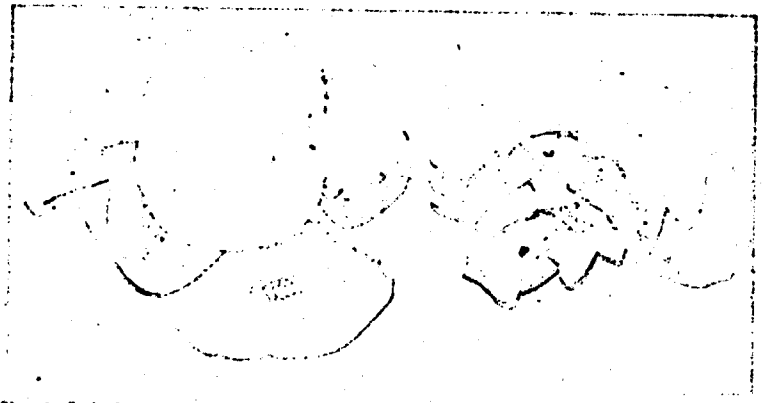

FIG. 1. *Left.* Macroscopic appearance of liver of rat fed high-fat diet, showing multiple red infarcts 3 to 12 hr. after the endotoxin injection. *Right.* A similar liver in which the infarcted areas have been removed. The difference in the weight of the liver before and after removal of the lesions gives a fair indication of the severity of the thrombotic phenomenon.

on a group basis between the serum triglycerides and the survival time on the pooled data of experiments 1, 2 and 3. The survival time was also related to the serum cholesterol, but the correlation coefficient r was only -0.69.

Experiment 4 (table 5). As in experiment 2, the highest serum cholesterol triglyceride level at both the 10- and the 18-week period was observed in rats fed cacao butter; in these animals, the most severe atherosclerotic lesions were also noted. In contrast to this, the group fed olive oil presented, at both periods, a degree of lipemia comparable to the butter-fed rats, but the lesions were incomparably less severe in the rats fed olive oil. Corn oil, the fat with the lowest lipemic effect, was also the least atherogenic.

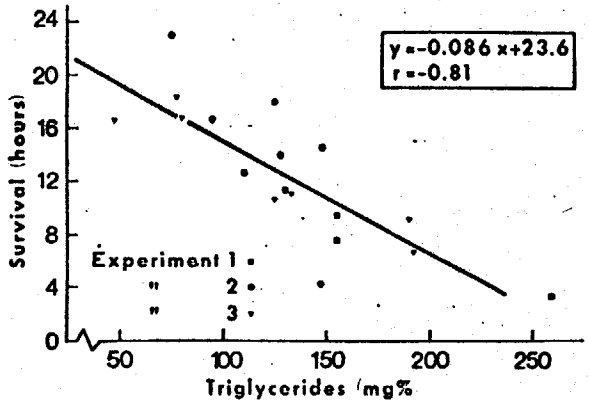

FIG. 2. The relationship, on a group basis, between the serum triglyceride level and the survival time in endotoxin-injected rats. The results are the pooled data of experiments 1, 2 and 3.

TABLE 5

Experiment 4: influence of dietary fats on lipemia and atherosclerosis

Results = Mean ± S.E. Total duration of the experiment 18 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water): casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Dietary Fat	No. of Autopsied Animals	Cholesterol		Triglycerides		Atherosclerosis (Scale 0-3)
		Week 10	Week 18	Week 10	Week 18	
		mg/100 ml		*mg/100 ml*		
Cacao butter	4	1136 ± 27	2775 ± 654	217 ± 14	582 ± 175	2.3
Butter	5	459 ± 11	2001 ± 696	124 ± 10	413 ± 160	1.7
Coconut oil	6	711 ± 9	516 ± 36	156 ± 25	117 ± 12	1.5
Olive oil	6	680 ± 203	1621 ± 342	188 ± 56	309 ± 70	0.3
Corn oil	6	301 ± 12	383 ± 34	57 ± 3	76 ± 9	0.05

DISCUSSION

The pathogenesis of our experimental model in which voluminous occlusive thromboses located almost exclusively in the hepatic veins are produced in rats fed a high-fat diet is not yet completely elucidated. Nevertheless, for years now, from one series of experiments to another, a continuously high incidence of multiple, red hepatic infarcts, resulting from the complete occlusion of the hepatic veins, has been consistently observed in rats fed the butter diet. We had already noted[9] that fatty infiltration of the liver has no bearing on the site of the thrombus formation. Despite the heavy fatty infiltration, it can also be added that liver protein synthesis in these animals is no different from that of rats fed laboratory chow.[22] In addition, we have observed in experiments not yet terminated that administration of epinephrine or norepinephrine instead of endotoxin to rats fed a high-fat diet will also initiate the formation of a voluminous thrombotic phenomenon, although in such cases the thrombi are located in the left atrium.

The most simple explanation for the hepatic veins as the primary site of thrombosis after an injection of Gram-negative bacteria endotoxin is that, in rat, the affinity of the liver for the bacterial toxins appears to be much greater than that of any other tissue.[23] It has also been observed[23] that, after the liver, the tissues retaining the largest quantity of toxins and the most frequent site of thrombosis are the spleen and the lungs. However, at the dosage of endotoxin utilized in this experiment, occlusive thrombi are found almost exclusively in the liver; therefore, from a practical point of view, only this organ has been considered in evaluating the severity of the thrombotic process.

The results of experiment 1 in this work confirm our previous results[12, 13] in that palmitic acid is much more hyperlipemic than stearic and caprylic acid, a finding that is also concordant with results obtained in man.[14, 16] In contrast to this, our observations with myristic and lauric acids are markedly different from those reported in man.[14, 16] Myristic acid induced a lipemia comparable to that induced by caprylic acid, and lauric acid was by far the least hyperlipemic of the five saturated fatty acids examined here. Of course, these results were obtained with purified free fatty acids and, as previously discussed,[13] the digestibility of the corresponding triglyceride can differ markedly from that of the acid and could explain the discrepancy in the results obtained when fats are used. However, among the fats studied in this series, only coconut oil contained large amounts of lauric and myristic acids. The results of experiment 3 showed that even in large-scale experiments, the cholesterol was the same in animals fed butter as in those fed coconut oil, and the triglycerides were lower in the latter group. Therefore, under our conditions, lauric and myristic acids cannot be highly hyperlipemic. From acute experiments with coconut oil in the dog,[24] it was concluded that saturated fatty acids of 12 and 14 carbon atoms had the most powerful effect in raising serum cholesterol, a finding that could also be applied to humans.[15]

However, the reason for this is far from clear, inasmuch as it has been reported that coconut oil decreases cholesterolemia in hyperlipemic patients[25] and that coconut oil fed to rabbits induced hypercholesterolemia only for 3 months,[26] an indication of the difficulties in drawing conclusions from short-term experiments. Under our conditions, the dietary feeding lasts for only 6 to 18 weeks, but in 6 to 10 weeks, owing to the type of diet, the animals are predisposed to the development of large thromboses that would normally occur in man, either in the coronary artery or in the veins,[27] only after 40 years of age. Therefore, in determining the value of our experimental model for reproducing human conditions, the results so obtained will have to be compared mainly with epidemiologic studies in man. As an example, it has been reported that the diet of the Atiu-Mitiaro islanders[28] is high in coconut. The serum cholesterol of the 40- to 49-year-old men is 235 mg per 100 ml, a value certainly no higher than those observed in man in North America, the Netherlands and Finland, where butter and dairy products certainly represent the largest portion of the dietary fat.[29]

It is known that olive oil has no cholesterol-lowering effect,[14] and in our experiments with oleic acid its cholesterolemic effects were not much different from those of stearic acid.[12, 13] In animals fed olive oil as the sole fat, the resulting cholesterolemia was slightly higher than with butter. Bovine tallow, which is much richer in oleic acid than butter, yielded a similar result. In all the experiments presented here, the lowest lipemia was recorded after feeding horse tallow (experiment 3), corn oil (experiment 2) and chicken tallow, horse tallow being richer in linolenic acid, and the two others in linoleic acid. In previous experiments,[12] these two acids were found to be the least thrombogenic and may have even antagonized the damaging effect of stearic and palmitic acids. The marked reduction in thrombogenicity that resulted from substitution of 6 per cent of the butter by corn oil in experiment 2 also suggest an antagonizing effect of linoleic acid.

With regard to thrombosis, our aim was to verify whether dietary fats with a high content in stearic and palmitic acids, the acids found to be the most thrombogenic in previous experiments,[12, 13] would be also the most thrombogenic. In experiment 2, cacao butter, by far the fat richest in stearic acid, was in fact the most thrombogenic; corn oil, the poorest in this fatty acid, was the least thrombogenic. These results with fats confirm those obtained with free fatty acids in previous[12, 13] and this series, in that only palmitic and stearic acids appeared to be highly thrombogenic. These data are also concordant with studies *in vitro* indicating that the long-chain saturated fatty acids are the most effective in aggregating blood platelets. In addition, in this experiment, with a few selected fats markedly different in the type of fatty acids they contained, almost all of the parameters examined (cholesterol, triglycerides, clotting time) appeared to be related to the severity of thrombosis.

The atherosclerosis experiment also confirms our earlier results that the most thrombogenic fatty acids are also the most atherogenic, independently of their

lipemic effect. Olive oil, which is more hyperlipemic than coconut oil, appears to be much less atherogenic and also much less thrombogenic. We had already found that oleic acid was almost no more thrombogenic[13] than linoleic acid, and only slightly more atherogenic.[12, 13] However, we also found that purified triolein was as thrombogenic as tristearin.[13] Under the present conditions, the only interpretation that can be offered for the surprisingly low thrombogenicity of olive oil and mostly bovine tallow is that oleic acid, whether in its free or in its triglyceride form, is not thrombogenic. Another surprising result is the thrombogenicity of arachis oil despite its high content in oleic and linoleic acid. This, however, confirms exactly what we had previously reported[11] and it is possible that, in this fat, the small quantity of long-chain fatty acids could be very active under the present conditions.

The most deceptive result is perhaps the fact that in experiment 2 a close relationship is obvious between various parameters and the severity of thrombosis, whereas in experiment 3, where a large number of various fats were used, none of the parameters examined could really be utilized for predicting the severity of thrombosis, a result similar to that already reported with various fatty acids.[12, 13] Of course, large thromboses cannot be initiated by endotoxins in animals with a serum cholesterol level below 200 mg and with a clotting time longer than 180 sec. On the basis of a large scale experiment, however, all that can be said is that these parameters represent no more than risk factors and by no means serve to predict closely the thrombotic tendency. From our experience with the addition of free fatty acids to the diet, these results are not surprising, because we have observed that the thrombogenicity of the different fatty acids does not necessarily parallel their effect on serum cholesterol or triglycerides. If, however, as suggested by these experiments, the thrombotic tendency is mostly due to the effect of some fatty acids on blood platelets, the parameters examined, except to some extent the clotting time, do not indicate this. Nevertheless, although the plasma clotting time as performed under our conditions is a highly constant and reliable determination, it is much more affected by the environmental conditions of the animal than the thrombotic phenomenon itself and, therefore, does not appear to constitute the ideal test.

Under the present conditions, it seems clear that neither the origin of a fat nor its degree of saturation governs its lipemic, thrombogenic or atherogenic effects. The type of fatty acids it contains is probably responsible for the above-mentioned effects; but due to the number of fatty acids involved and the various interactions observed to occur with such dietary feeding,[30] many more experiments will need to be done before we can completely predict the lipemic and thrombogenic effects of certain fats or fat mixtures. Also, with so many data to deal with, the help of a computer will probably be necessary, as other investigators have realized under similar conditions.[30] Another source of error is that, except for four samples, the composition in fatty acids of the fats used here has not been determined and could be slightly different from this reported in table 3.

As regards the mortality rate and the survival time, in almost none of the present or previous experiments did we find it to be related to the severity of the occlusive thrombosis. At least on a group basis, the survival time can be correlated with the degree of lipemia, as previously observed,[10] and the mortality is probably attributable to shock. In rabbits treated by injection with bacterial endotoxin, it was also reported that the mortality rate was related to the triglyceride level.[31]

From a practical point of view, it seems essential to evaluate to what extent the present experimental model in the rat yields results that are comparable to those obtained in man and also the degree to which it could help to clarify the pathogenesis of large thrombosis and coronary disease in man. Under our conditions, after cacao butter, which has also been found to be highly atherogenic in the rabbit,[26, 32] the most thrombogenic fat is butter and, therefore, probably the other dairy products also. Dairy products represent a large portion of the dietary fat of the highly civilized countries with a high rate of coronary disease. Coconut oil, although able to induce a degree of cholesterolemia similar to that of butter, does not seem to be highly thrombogenic in man[28] or under our experimental conditions. Olive oil, like oleic acid a nonthrombogenic fat, is largely used in the diet of countries such as Italy or Greece where the incidence of coronary disease is not as high as in northern Europe and North America.

These are only a few examples of the relationships that should be underlined and, it is hoped, that will stimulate fruitful collaboration between the experimental approach on the animal and epidemiologic studies in human.

SUMMARY

Among the more common saturated fatty acids, palmitic acid appears to be the most hyperlipemic when added to the basic high-fat diet of rats. Under these conditions, stearic and especially lauric acid seem to have much less effect on both the cholesterol and triglyceride serum levels. However, the rats fed the long-chain fatty acids, palmitic and mostly stearic acids, presented the most severe phlebothromboses, initiated in these rats by the intravenous injection of a *S. typhosa* endotoxin and resulting in multiple red hepatic infarcts.

Fats richer in stearic acid, such as cacao butter and butter, are also much more thrombogenic than lard and corn oil, which contain less stearic acid and much more linoleic acid, probably an antithrombogenic fatty acid.

Among a large number of fats fed to rats under comparable conditions, the results obtained were not as clear because butter was the most thrombogenic and olive oil and bovine tallow, despite a high content in stearic acid, the least thrombogenic. The low thrombogenicity of these latter fats, despite a marked hyperlipemic effect, is probably due to their high oleic acid content. However, the thrombogenicity of a fat is probably the result of multiple fatty acid interactions and should be further analyzed.

Only with a limited number of fats could the lipemia or the plasma

clotting time be utilized to predict the thrombotic tendency of the animals. But in large-scale experiments, serum cholesterol and triglycerides, as well as the plasma clotting time, could be considered merely as risk factors. In contrast to this, there was a highly significant correlation between the survival time of the endotoxin-treated animals and their serum triglyceride level.

The effects of a few selected fats on the production of aortic fatty streaks in the rat seem to be related to the thrombogenic effect and also to the composition in fatty acids. Under the conditions of this study, neither the degree of saturation of a fat nor its origin (animal, vegetable) appears to be of any use for predicting its lipemic, thrombogenic or atherogenic effects.

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Metabolic Products of Yeast Fermentation: Organic Acids, Isolation, Separation and Characterization

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While today's investigations into many aspects of brewing chemistry proceed, it is perhaps wise to take note of unsolved problems. We know that changes occurring in proteins and enzymes have been more extensively studied than those of lipids.

It is apparent that wort and beer fatty acids are affected by malt processing conditions (1,2,22). However, the following questions are raised:

1. The role of the subsequent lipids in wort and beer is still unknown; further study might establish relations with raw materials composition and variations in brewing and fermentation.
2. Few data are available concerning heritability of fatty acid composition in beer. This poses a series of questions regarding reactions involved in plant utilization and during beer processing.
3. No conclusion could be drawn from their implication in flavor stability; even in traces, they may influence the taste and aroma, thus affecting the organoleptic qualities of beer.
4. There is the possibility that some degradation of liberated acids within the beer may well have taken place during storage.

A study of available literature relating to organic acids present in beer showed that the results usually were based on total acids or partially volatile acids, with contradictory statements on the component acids.

A previously reported investigation (18) discussed briefly the acid fraction in beer. As an extension of that study this paper presents preliminary results of acids patterns during beer processing.

This work was undertaken with a view toward:

1. extending knowledge of the composition of beer,
2. exploring gas-chromatographic techniques for separation and characterization of organic acids,
3. understanding the metabolism of organic acids during barley, malt, and beer processing,
4. determining the fate of these acids at several stages of processing and aging, and
5. assessing the effect of their presence in varying proportions on beer flavor.

The nature of beer flavor intrigues brewers and chemists; however, the complexity of organic acids with its inherent difficulties have generally frustrated their efforts.

In tasting beer we perceive its slightly acid character produced by the buffering effect of primary phosphates, by the amino acids and by other organic acids.

Organic acids in beer quantitatively constitute a very minor fraction but are comprised of a variety of classes: saturated and unsaturated, mono- and polybasic acids including hydroxy, aldo and keto acids, of the aliphatic as well as a few of the aromatic series (Table I).

Experimental

The fundamental steps in any analysis are isolation, concentration, separation and characterization or identification.

Table I.
Organic Acids in Wort and Beer

Of Straight Chain Acids		Of Branched Chain Acids
Saturated	**Unsaturated**	**Saturated**
Monocarboxylic:		
$C_1 - C_{18}$	Hex-2-Enoic	2-Methyl Propanoic (iso-butyric)
Benzoic	Hex-3-Enoic	2-Methyl Butanoic
Phenyl Acetic	Hept-3-Enoic	3-Methyl Butanoic (iso-valeric)
Glycolic (hydroxy ethanoic)	cis-Octadec-9-Enoic (oleic)	4,4-Dimethyl Butanoic (iso caproic)
Lactic (2-hydroxy propanoic)	trans-Octadec-9-Enoic (elaidic)	2-Hydroxy-3-Methyl Butanoic (2-hydroxy iso-valeric)
2-Hydroxy-Butanoic	n-Octadec-9,12-Dienoic (linoleic)	2-Hydroxy-4-Methyl-Valeric
2-Hydroxy-Hexanoic	n-Octadec-9,12,15-Trienoic (linolenic)	2-Hydroxy-3-Methyl-Valeric (2-hydroxy iso-caproic)
3-Hydroxy-Octanoic		2-Oxo-3-Methyl-Butanoic (α-keto-iso valeric)
Salicylic (hydroxy benzoic)		2-Oxo-4-Methyl-Valeric (α-keto-iso caproic)
2-Hydroxy-3-Phenyl Propionic		2-Oxo-3-Methyl Valeric (α-keto-β-methyl valeric)
p-Hydroxy Phenyl Acetic		
Glyoxylic (oxo-ethanoic)		
Pyruvic (α-keto propionic)		
α-Keto Butyric		
Aceto Acetic (β-keto butyric)		
Levulinic (γ-keto valeric)		
Oxalo-Acetic		
α-Keto Glutaric		
Di- and Tricarboxylic:		
Oxalic (ethandioic)	Fumaric (trans-ethylene 1,2-dicarboxylic	
Malonic (propandioic)	Citraconic (cis prop-1-ene 1,2-dicarboxylic)	
Succinic (ethane-1,2-dicarboxylic)	Mesaconic (trans prop-1-ene 1,2-dicarboxylic)	
Malic (2-hydroxy butanedioic)	Aconitic (cis-1,2,3-propene tricarboxylic)	
Tartaric (2,3-dihydroxy butanedioic)		
Citric (2-hydroxy-1,2,3-propane tricarboxylic)		
Isocitric (1-hydroxy-1,2,3-propane tricarboxylic)		

Isolation of Acids

To avoid a autoxidation, oxi enzyme action interaction, whic information, a gr necessary during t flavor isolation.

A. Volatile Acic

The most co isolating the volati wort or beer is b normal or reduced on a trap cooled wi under a variety of c methods were deve (Fig. 1).

Method 1.

Beer was vacu temperature for 45 contained the ne and some free fatty trapped. By adjus residue to 2.2 an steam, a condens volatile fatty acids v

Method 2.

The beer, adju addition of 10% so

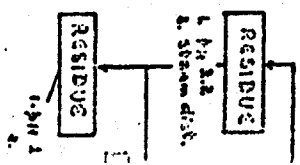

Figure 1.

Isolation of Acids

To avoid artifacts arising from autoxidation, oxidation, metal catalysis, enzyme action, heat, or chemical interaction, which limit the value of information, a great amount of skill is necessary during the very critical phase of flavor isolation.

A. Volatile Acidic Fraction

The most convenient method for isolating the volatile acidic fraction from wort or beer is by steam distillation at normal or reduced pressure and collection on a trap cooled with ice. Following trials under a variety of conditions, two general methods were developed for this purpose (Fig. 1).

Method 1.

Beer was vacuum distilled at low temperature for 45 minutes. The distillate contained the neutral volatile fraction and some free fatty acids; carbonyls were trapped. By adjusting the pH of the residue to 2.2 and then distilling with steam, a condensate which contained volatile fatty acids was obtained.

Method 2.

The beer, adjusted to pH 8.3 by addition of 10% sodium carbonate, was steam distilled for 30 min. The neutral volatile fraction was separated. Extraction of the residue with ether removed the neutral non-volatile fraction. The alkaline solution was adjusted to pH 1.0 and steam distilled again. The condensate contained the volatile acids.

Recovery of known amounts of volatile acids.

Fatty acids in diluted alcoholic solution were added individually, or in combinations of two, three or more, to the distillation flasks containing water or beer. The determinations were carried out in duplicate and the results are shown in Table II.

B. Non-volatile Acidic Fraction

After steam distillation of the volatile acidic fraction in both methods (1 and 2 above), the non-volatile acids were recovered by continuous extraction with ether for 24 hours of the residue adjusted to pH 1.0.

Concentration of Volatile Acids

For the concentration of the volatile acidic fraction two procedures were employed.

Procedure A.

Since none of the numerous methods proposed for the determination of total

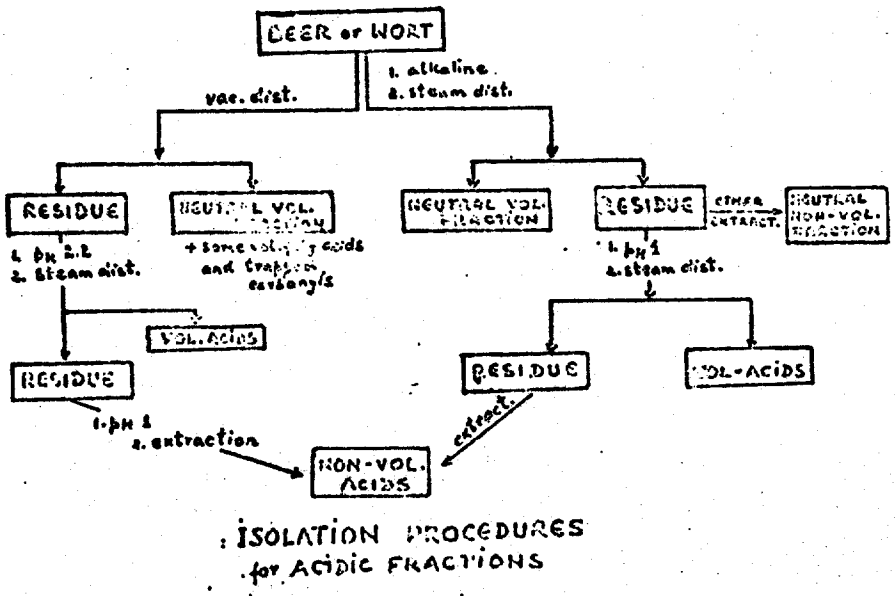

Figure 1.

Isocitric (1-hydroxy-1,2,3-propane tricarboxylic)

Table II.
Recovery of Known Amounts of Organic Acids

Organic Acid Added	cc. of 0.01 N equivalents	Mean Recovery	% Total Acid Recovered 1st 50 cc.	2nd 100 cc.	3rd 100 cc.	4th 150 cc.
Acetic	25	101	34	28	25	14
Acetic-Butyric	25:20	98.4	42	27	18	11.4
Acetic-Butyric-Caproic	25:20:7.7	97.5	49	23	15	10.5
Acetic-Propionic-Butyric-Caproic-Caprylic	25:26:21:3.8:6	99.2	48	26	17	8.2

volatile acids appeared to have any pronounced advantage, the technique adopted in this study has been restricted to the determination by titration. The titrations were carried out with phenolphthalein indicator using diluted NaOH (0.01 - 0.05N). All replicate determinations gave consistent results (Table III). After conversion to their salts they were concentrated and dried under vacuum.

Procedure B.

The condensate was saturated with sodium chloride and the volatiles were extracted with ether or petroleum ether. In an attempt to remove non-acidic water soluble components, the acids were extracted repeatedly with ether after acidification, dried over sodium sulfate, concentrated and kept in the freezer.

Formation of Derivatives of Acids

A. Ammonium Salts

The sodium salts were extracted into butanol after acidification with sodium bisulfate and converted to ammonium salts by neutralization to pH 7.

B. Hydroxamic Esters

Hydroxamic esters can be prepared from methyl esters, from free acids or from sodium salts.

For the preparation from sodium salts, thionyl is added to the salts and the mixture allowed to stand for 15 min. Absolute ethanol is then added and after 30 min. the resulting mixture is treated with water to decompose any excess of thionyl chloride. The formed esters were extracted with ether. The dried and concentrated ether extract was mixed with hydroxylamine solution and, after 30 min. standing at 25°C., acetic acid was added. The precipitate that formed was filtered and the ether removed under reduced pressure. The residue contained the hydroxamates.

C. Methyl Esters

Because the esters are relatively stable towards heat they can be distilled without decomposition. For this reason fatty acid mixtures usually are converted to their esters for separation and identification.

Methyl esters of the organic acids were prepared by three methods; bearing in mind our preference for rapid reaction, quantitative yield and convenience.

Method 1. By the use of a BF_3-MeOH (10% W/V) mixture. (14)

Method 2. By the use of diazomethane. (21)

Method 3. The sodium salts of the acids were heated for 30 min. in alcohol

Table III.
Volatile Acidic Fraction in Wort and Beer

	Volatiles (as Acetic Acid) mg./Liter Sample 1	Sample 2	Sample 3	Sample 4
Wort	36±0.3	37.8±0.3	34.5±0.3	42±0.3
Beer	174±0.6	165±0.6	195±0.6	186±0.6

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Paper Chromatography

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with a small excess, above equivalent quantities, of sulfuric acid.

The degree of methylation with each of these methods was checked by thin-layer chromatography using Silica Gel G and developed with a petroleum ether, diethyl ether, glacial acetic acid mixture (90:10:1 v/v/v/). The degree of methylation proved to be very satisfactory.

Separation and Identification

The methods employed for separation and characterization of isolated and concentrated acids and/or their derivatives include paper chromatography, thin-layer chromatography, and gas liquid chromatography. Also a purification step on a glass column containing silicic acid, using the procedure of El-Gharbawi et al. (7), was sometimes employed.

Paper Chromatography

In order to check the identify of each component paper chromatography primarily was used. Sodium salts were developed in a solvent system of diethyl ether, acetic acid, water (13:1:1 v/v/v) and ammonium salts in a n-butanol, 1.5 N NH_3 (1:1 v/v) system (14). Alkaline bromphenol blue solution revealed sodium salts as yellow spots on blue, while the acidic showed ammonium salts as blue spots on a yellow background. The disadvantages of detection using base-acid indicators are due to low sensitivity, susceptibility to atmospheric carbon dioxide and solvent system components, as well as color instability.

The next step was to try the hydroxamates, which play a major role in the study of Acyl Coenzymes A. Their detection did not cause any major difficulties. Three solvent systems were investigated: benzene, formic acid, water (1:1:1 v/v/v); n-butanol, dimethylformamide, water (1:6:8 v/v/v), and ethyl acetate, tetrahydrofurane, water (1:6:8 v/v/v). The latter, following the method of Goldschmidt et al. (8) on acetylated paper, proved to be superior to the other two in separating C_2 - C_{18} hydroxamates. The spots were made visible as red complexes on yellow by spraying with $FeCl_3$ solution in n-butanol. For higher aliphatic and unsaturated acids sharp resolution of C_{10} - C_{18} was obtained on paper impregnated with hydrocarbons, using acetic acid for development (19). The resolution of the so-called critical pairs, i.e. saturated and unsaturated acids with identical R_f values was accomplished by two dimensional chromatography or by direct hydrogenation on paper (12).

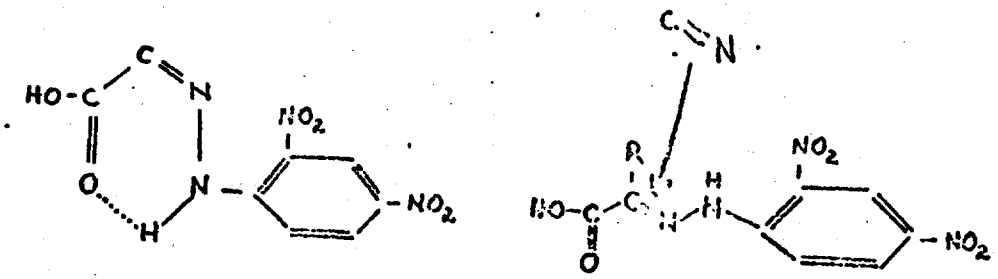
The keto acids, which are also compounds of biochemical importance, being unstable and susceptible to interference by other compounds of the mixture, were analyzed as 2,4 dinitrophenyl hydrazones. The most popular mobile phases used were: n-butanol, ethanol, water (4:1:5 v/v/v) (6); tert-amyl alcohol, ethanol, water (5:1:4 v/v/v), on paper impregnated with phosphate buffer (11). As colored compounds the DNP hydrazones were visible on the paper; spraying with dilute sodium hydroxide intensified their color.

It should be noted that pyruvic and glyoxylic acids each showed two spots. Two structures are suggested, the syn- and anti-forms caused by isomerism of the phenylhydrazone itself (Fig. 2). The spots were cut out and dissolved in N NaOH. Spectrophotometric analysis showed different curves for each spot.

Additional spots appeared on the DNP hydrazone papers that were estimated as α-keto-isovaleric, α-keto-isocaproic, α-keto-butyric, and α-keto-β-methyl-valeric acid. Several more barely visible spots, as yet uncharacterized, and probably as a result of polymerization (pyruvic acid) or decarboxylation (oxalo-acetic to pyruvic acid), or perhaps artifacts, also appeared.

To separate the non-volatile acids their solutions were chromatographed using the following systems:

a) n-pentylalcohol, 5M formic acid (1:1 v/v),
b) n-butanol, ethanol, 0.5 N NH_3 (7:1:2 v/v/v),



R = H - Glyoxylic
R = CH_3 Pyruvic

Figure 2.

c) n-butanol, water, acetic acid (4:1:1 v/v/v),
d) n-butanol, water, propionic acid (10:7:5 v/v/v).

Very good resolution was attained with system "d". Detection was made with silver nitrate (acids appeared as white spots on a brown background) or was revealed by a blue fluorescence after heating the chromatogram at 150-160°F. R_f values obtained for the unknowns were almost identical to those of di- and tricarboxylic acids.

Table IV.
Paper- and TLC of Organic Acids

	NH_4-Salts	Hydroxamates Paper	Hydroxamates TLC	Free Acids Without Hydrogenation	Free Acids With Hydrogenation	Esters TLC	2,4 Dinitro Phenyl Hydrazones
Metanoic	+	+	+				
Ethanoic	+	+	+				
Propanoic	+	+	+				
n-Butanoic	+	+	+				
2-Methyl Propanoic	+	+	+				
n-Pentanoic	+	+	+				
3-Methyl Butanoic	+	+	+				
n-Hexanoic	+	+					
n-Octanoic	+	+					
n-Decanoic		+					
n-Dodecanoic		+		+			
n-Tetradecanoic		+		+			
n-Hexadecanoic		+		+		+	
n-Octadecanoic		+		+		+	
n-Octadec-9-Enoic (oleic)		+		(+)	+	+	
n-Octadec-9,12-Dienoic (linoleic)		(+)		(+)	+	+	
n-Octadec-9,12,15-trienoic (linolenic)				(+)	+		
Glyoxylic Syn							+
Glyoxylic Anti							+
α-Keto-iso-Valeric							+
α-Keto-β-Methyl-Valeric							+
α-Keto-iso-Caproic							+
α-Keto Butyric							(+)
Levulinic							+
Oxalo-Succinic							+
Oxalo-Acetic							+
α-Keto Glutaric							+
Pyruvic Syn							+
Pyruvic Anti							+

+ compound detected
(+) mixture of sat. and unsaturated
(+) tentatively detected

Table V.
Paper- and TLC of Non-Volatile Org. Acids

	Paper Chromatog. Solvent Systems			Thin-Layer Chromatog.			
	A	B	C	a	b	c	d
Oxalic	+	+	+	+			
Malonic	+	+	+	+			
Succinic	+	+	+	+	+	+	+
Glycolic	+	+	+				
Lactic							+
Malic	+	+	+		+	+	+
Citric	+	+	+		+	+	+
Iso-Citric	+	+	+		+	+	+
Tartaric						+	
Glyoxylic	+	+	+				
Pyruvic							+
Oxalo-Acetic	+	+	+		+		
Oxalo-Succinic	+	+					
α-Keto Glutaric	+	+	+		+		+
Fumaric	+	+	+		+		+
Aconitic		+	+		+		

A: n-Butanol-Water-Acitic Acid (4:1:1 v/v/v)
B: n-Butanol-Water-Propionic Acid (10:7:5 v/v/v)
C: n-Butanol-Water-Abs.Ethanol (10:2:1 v/v/v)

a: Silica Gel G
b: Cellulose Powder
c: Silica Gel H
d: Cellulose MN 300

Thin-Layer Chromatography

The separation and detection of organic acid fractions isolated from beer and wort was investigated on Silica Gel G and H plates, on Cellulose M N 300, on cellulose powder and on thin sheets of cellulose. The hydroxamates of monocarboxylic acids up to C_5 were separated on thin sheets of cellulose using an alcohol-ammonia solvent (3). On Silica Gel G plates impregnated with silicone oil and sprayed with silver nitrate, common critical pairs - linoleate/myristate and oleate/palmitate - were fully resolved with 95% aqueous methanol and detected with phosphomolybdic acid (16).

The dicarboxylic acids, oxalic, malonic, and succinic, were separated on Silica Gel G with a n-butanol, xylene, phenol, formic acid, water system (10:70:30:8:2). Detection was achieved with alkaline bromcresol purple (17).

Lactic acid and some citric cycle acids, intermediates of the Krebs cycle, were separated by thin layer chromatography using cellulose powder. The plates were developed with a anhydrous diethyl ether, formic acid, distilled water system (7:2:1) and the separated acids detected with bromcresol purple (15).

Citric, isocitric, malic, succinic, and tartaric acids were separated on Silica Gel H using a water saturated diethyl ether, formic acid system (7:1) and detected with ammonium molybdate (20).

Two dimensional thin-layer chromatography on Cellulose M N 300 using a 96% ethanol, water, 25% ammonium hydroxide system (100:12:6) for the first development and for the second dimension a propanol, eucalyptol, formic acid, water system (50:50:20:2), separated lactic, fumaric, pyruvic, succinic, α-ketoglutaric, malic and citric acids. The spots were made visible with aniline-ribose (9). Paper- and thin-layer chromatography results are shown in Tables IV and V.

Gas-Liquid Chromatography

Aside from paper- and thin-layer chromatography, organic acid analysis can be performed using other techniques; however, most of them have limited sensitivity, reproducibility, and applicability to large groups of acids, and are time consuming. In this study, requiring a rapid, reproducible technique, we applied gas-liquid chromatography.

We first analyzed the organic acids of wort and beer as methyl esters. Each fraction, volatile and non-volatile, was studied with several stationary phases coated on Chromosorb W, Diatoport W, and Diaport S, screened to 45-60 to 80-100 mesh as packings. The liquid phases for the resolution of the free acids and/or their methyl esters included SE-30, SE-52, Porapak Q with 2% H_3PO_4, Tween 80, Carbowax 20M, Apiezon L, DEGS, and DEGA.

Extensive use was made of three columns, namely, DEGS and Apiezon L for the steam distilled fraction and Carbowax 20M for the non-volatile fraction. DEGS and Apiezon L represent a contrast in polar-nonpolar structure and aid identification as well as serving as a criterion of satisfactory performance.

Esters used for test mixtures,

calibration curves and internal standards were obtained commercially and appeared highly pure, yielding only one peak each on the chromatograms.

In referring to column performance some compromise will be required. Each column has merits and limitations. On the Tween 80 column 8 minutes was necessary to carry out an analysis, however, the separation of propionic and isobutyric is imperfect. Porapak Q eliminates the disadvantage of Tween 80, and can be used in routine analysis, but is limited to fatty acids with no more than 6 carbon atoms. Apiezon L capillary did not appear to separate the C_{18} diene from triene, while the polyester column revealed a slight but consistent inflection in front of the oleate peak. Areas of the peaks were computed and the quantities of individual components were determined as percent of the total peak area. On the basis of the preceding calibration studies, based on C_{17} recoveries, quantitative analytical data were obtained from wort and beer.

Results and Discussion

The use of paper -, thin-layer, and gas liquid chromatography enabled us tentatively to identify a series of organic acids. Thus far 64 acids were revealed. Identifications were based on three methods:

1. On the coincidence of R_f, known - unknown, on paper- and thin-layer chromatograms.
2. On the coincidence of retention times by comparison with known standard materials run either immediately before or immediately after the test samples on several different gas chromatographic columns.
3. On a plot of carbon chain lengths against the logarithms of retention times yielding a straight line; this method when used alone is not too reliable in application to a complex mixture.

The results for the volatile acids are shown in Table VI. The data indicate that the nature of the steam distilled organic acids from wort and beer is similar but with differences in quantities. The total amount of volatile acids, always greater in beer than in wort, seems to be due primarily to acetic acid, followed by lesser quantities of formic, caproic, caprylic, propionic and decanoic acids. Such components presumably occur as dissociable free fatty acids or as salts.

Table VI.
GLC-Data
(Monocarboxylic Acids)

	Wort (ppm.)	Beer (ppm.)
Formic	1	30 ± 5
Acetic	15 ± 5	100 ± 10
Propionic	0.4 ± 0.1	3.6 ± 0.5
iso-Butyric	0.3 ± 0.1	1.9 ± 0.5
Butyric	0.1 ± 0.05	0.6 ± 0.3
iso-Valeric	0.9 ± 0.2	1.6 ± 0.1
Valeric	0.5 ± 0.1	0.2 ± 0.1
Caproic	0.85 ± 0.15	4.15 ± 1.5
Enanthic	0.7 ± 0.2	0.5 ± 0.15
Caprylic	0.3 ± 0.1	7.0 ± 1.5
Pelargonic	0.45 ± 0.15	0.4 ± 0.2
Capric	0.2 ± 0.1	2.4 ± 0.5
Undecanoic	trace	1.4 ± 0.5
Lauric	0.2 ± 0.1	0.7 ± 0.35
Tridecanoic	trace	1.1 ± 0.2
Myristic	0.25 ± 0.05	0.25 ± 0.15
Palmitic	1 ± 0.2	0.45 ± 0.05
Stearic	0.45 ± 0.25	0.3 ± 0.1
Oleic	0.15 ± 0.05	0.3 ± 0.2

Extensive studies during fermentation indicated that a considerable change took place. After a lag or slight rise during the initial 12 hour period of fermentation, the 24 hour levels rose fairly rapidly (Table VII). The level of these volatile acids increased steadily and, by the end of the 4th day of fermentation, reached their highest peak.

The data indicate that the principal free acids of the beer are straight chain acids of even carbon atom number C_2 through C_{10}. It seems likely that they

Table VII.
Level of Volatile Acids
During Fermentation
(ppm.)

	Formic	Acetic	Propionic	Butyric	.....etc.
12 hrs.	1	10-15	0.4	0.1	
24 hrs.	7	70	3	1	

"HEAD TO TAIL" - CONDENSATION

(3) ACETYL CoA + 7 MALONYL CoA + 14 TPNH

$\longrightarrow$ PALMITIC ACID + $7CO_2$ + 8 CoA + 14 TPN

BETA-OXIDATION

(4) $R\text{-}CH_2CH_2COOH \rightarrow R\underset{OH}{\overset{H}{C}}\text{-}CH_2COOH \rightarrow R\underset{O}{C}\text{-}CH_2COOH \rightarrow RCOOH + 2CO_2 + H_2O$

β-hydroxy acid β-keto acid

Figure 3.

probably result from the normal fatty acid synthesis process either in the raw materials or in the yeast fermentation. This process undoubtedly involves an initial acetyl - coenzyme A reaction with bicarbonate and transfer to the sulfhydryl group of a soluble multienzyme system. Successive condensations of this malonyl enzyme complex with either acetyl - coenzyme A or with even carbon number acyl-soluble enzyme complexes would yield enzyme bound products increased by multiples of two carbon units (13). This biosynthetic mechanism being well worked out, the stoichiometry of the synthesis could be formulated as illustrated in Fig. 3 (3). All these reactions occur while the acid is still bound to the sulfhydryl group of the soluble enzyme, not being detached until the acid reaches a high carbon number.

The presence of large amounts of butyrate, caproate, and caprylate in beer suggests that

a. there is leakage from the enzyme system of a product of lower molecular weight (4) or
b. longer chain saturated acids initially produced are degraded - the classical beta-oxidation scheme is illustrated in Fig. 3 (4).

The source for straight chain odd carbon number acids is not known with certainty but it is suggested that their build-up could result with the same synthetase complex system but starting with a small amount of propionyl coenzyme A instead of acetyl coenzyme A. Propionic acid could arise from oxidation of propanal but the formic, enanthic, and pelargonic acids are produced by pathways at present still not clear.

The presence of isobutyric, isovaleric, and isocaproic acids among those acids isolated from beer suggest

a. Head to head condensation with subsequent loss of CO_2, resulting in branched chain carbon skeletons.
b. Perhaps equally probable, however, is an oxidation of the branched chain aldehydes involved in the amino acid-fusel oil mechanism in yeasts.
c. Horning et al. (10) have shown that acetyl CoA can be replaced by branched chain acyl CoA esters such as isobutyryl, isovaleryl, or 2-methylbutyryl. They suggested that the small amounts of branched chain fatty acids could be synthesized by this mechanism from the appropriate CoA esters derived from L-valine, L-leucine and L-isoleucine. The general metabolic pathway shown in Fig. 4 is postulated for biosynthesis of branched chain fatty acids. The lower part of the figure incorporates Horning's modification into the condensation equation. The α-keto

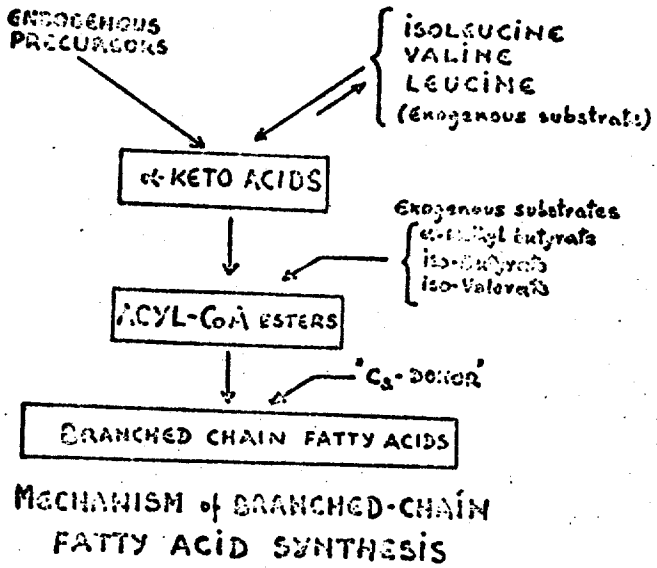


Figure 4.

acids, from which the acyl-CoA esters are derived, may be formed either from endogenous precursors or from exogenous amino acid substrates but the same α-keto acids may also form amino acids for further protein synthesis by the organism. A reduction in the amount of biotin in the yeast would be expected to reduce the supply of malonyl-CoA (C_2 donor in Fig. 4), since biotin is known to be an essential part of acetyl-CoA carboxylase. Probably one of the most important questions to be answered is why most living organisms produce straight-chain fatty acids rather than branched-chain fatty acids, even though the branched-chain amino acids, endogenously formed or exogenously supplied, are available to these organisms.

The non-volatile acids in wort and beer are also of considerable importance. The total amount of non-volatile acids was also greater in beer than in wort. This seems likely to be caused by increases in the amounts of pyruvic, lactic, succinic, and α-ketoglutaric acids produced in the course of fermentation (Table VIII). Glycollic acid seems not to occur in wort but is found in good amounts in beer. There is no significant change in the amounts of citric, malic, and fumaric acids. A small amount of oxalic acid could be detected in all beer samples, while mesaconic and levulinic, which could not be separately identified, are less in beer than in wort. It was established that certain non-volatile acids present in the wort disappeared during fermentation and were re-formed later in the course of this process.

On the basis of the amount of individual non-volatile acids a satisfactory characterization of beer or different fermentation procedures is possible only if the content of each acid in wort as well

Table VIII.
Non-Volatile Acids in Wort and Beer (GLC-Data)

	Wort (ppm.)	Beer (ppm.)
Mesaconic / Levulinic	12 ±1	9 ±1
Pyruvic	5 ±1	150 ±25
Fumaric	17 ±3	21 ±2
Succinic	14 ±2	47 ±3
Lactic	10 ±3	195 ±15
α-Keto Glutaric	19 ±2	30 ±5
Oxalic	7 ±2	3 ±1.5
Glycolic	-	29 ±3
Malic	53 ±3	50 ±4
Citric	89 ±5	95 ±6
Benzoic		
Phenyl Acetic		

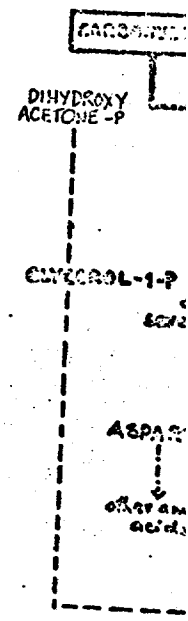


Figure 5.

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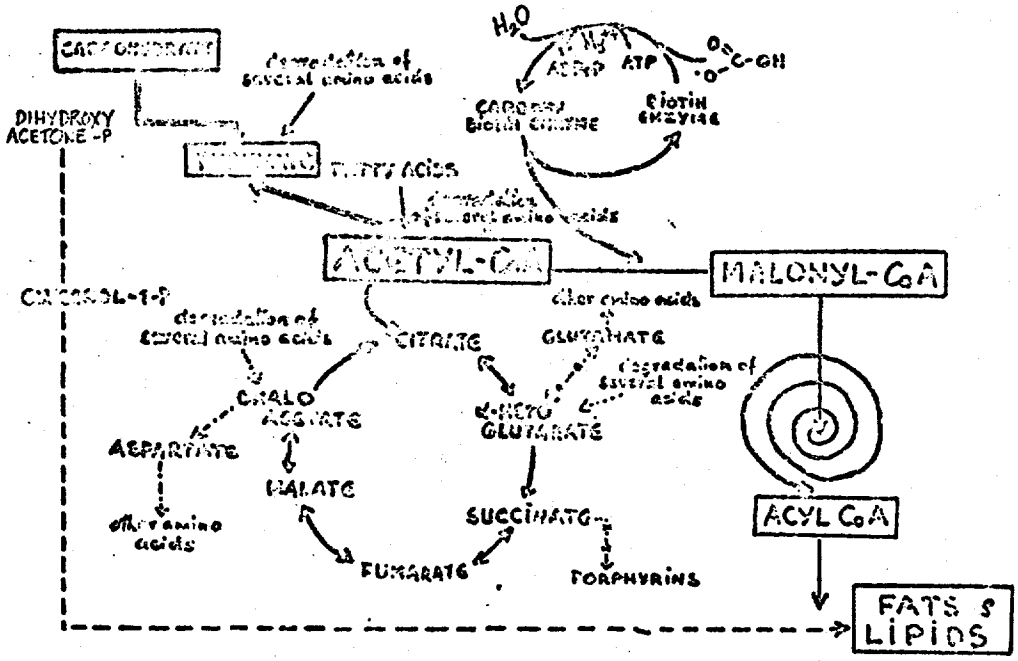

RELATIONSHIP of TCA-CYCLE to carbohydrate, lipid and protein metabolism.

Figure 5.

as in beer is taken into consideration. There is a very close relationship of the tri-carboxylic acid cycle to carbohydrates, lipids, and protein metabolism as indicated in Fig. 5. The fact that α-ketobutyric, α-ketoisovaleric, α-ketoisohexoic, and α-keto-β-methylvaleric acids could not be detected using a Carbowax 20M column is due to the relative instability of the keto acids. When chemical structure is established then the organoleptic properties must be determined, to give information about the contribution of organic acids to beer flavor.

The contribution of acids, especially those above propionic (C_4 to C_{10}), is certain to be of significance to the total aroma of beer produced during fermentation because of their very pungent and disagreeable odors even in very low concentration. Therefore, in order that the results as shown become meaningful, their relationship to the threshold taste level must be evaluated. Studies designed to establish organoleptic threshold values for these compounds, both individually and in combination, currently are in process.

Acknowledgment

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Hepatic Metabolism of 1-^{14}C Octanoic and 1-^{14}C Palmitic Acids

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Abstract

The hepatic metabolism of 1-^{14}C octanoic acid was compared with that of 1-^{14}C palmitic acid in male rats which were fed. After intraportal injection only 1/6 to 1/8 as much octanoic acid as palmitic acid was incorporated into hepatic lipids. In contrast, octanoic acid yielded two to four times as much water-soluble product as did palmitic acid. Similar, but even more impressive, differences between the incorporation of these fatty acids into hepatic lipids were observed in liver slices incubated with ^{14}C octanoate and ^{14}C palmitate. The oxidation of octanoate to CO_2 was more than 10 times as great as that of palmitate. With both substrates, triglycerides comprised almost half the labeled lipid recovered. However octanoate yielded a higher proportion of labeled, unesterified fatty acids and a lower proportion of labeled phospholipid and monoglycerides than did palmitate. Most of the ^{14}C recovered in hepatic lipids after incubation with 1-^{14}C octanoate was found in the carboxyl groups of long-chain fatty acids, suggesting that the latter had been synthesized from 2-carbon fragments formed from the oxidation of octanoate. In contrast, only a small fraction of the palmitate was elongated.

The similarities and differences between the metabolism of octanoic and palmitic acid in liver and intestine, and the possible nutritional significance of octanoic acid are discussed.

Introduction

TRIGLYCERIDES containing medium-chain fatty acids and those containing long-chain fatty acids differ with respect to their intestinal absorption and intramucosal metabolism (1–6). Thus long-chain triglycerides (LCT) undergo intraluminal hydrolysis to monoglycerides, fatty acids, and glycerol by pancreatic lipase before absorption, are reesterified to triglyceride within the mucosa, and are then exported as chylomicra via the lymphatics. In contrast, medium-chain triglycerides (MCT), containing principally octanoic and decanoic acids, can be absorbed intact, undergo hydrolysis within the mucosa by a mucosal lipase (4–6), and are then transported to the liver via the portal vein either as a salt or as an albumin complex (1–3).

Little attention has been given to a consideration of possible differences in the metabolism of medium- and long-chain fatty acids once they reach the liver. In the present report, evidence, based on both in vivo and in vitro experiments in the rat, is presented to show that medium-chain and long-chain fatty acids differ also with respect to their metabolism in the liver.

Methods

The male Sprague-Dawley type of albino rats (Charles River Rat Farm, North Wilmington, Mass.), weighing 180–220 g and maintained on Purina Lab Chow, were used in all experiments and were not fasted prior to use.

1-^{14}C octanoic acid and 1-^{14}C palmitic acid (New England Nuclear Corporation, Boston, Mass.) were purified by thin-layer chromatography prior to use. After dilution to the appropriate specific activity with unlabeled fatty acid (Hormel Foundation, Austin, Minn.) the mixture was dissolved in a small volume of diethyl ether and homogenized in a pH 7.4 Krebs-Ringer bicarbonate buffer (½ calcium), containing 10% Janel fatty acid-poor bovine albumin (Gallard-Schlesinger Chemical Manufacturing Corporation, Carle Place, New York), until the ether was evaporated.

Experiments in vivo

After anesthetization of rats with Nembutal the portal vein was isolated through a midline incision and injected in 1.0 min with 1.2 μMoles (2.4 μc) of either ^{14}C octanoic or ^{14}C palmitic acid in 1.0 ml of buffer-albumin solution. A 1-ml tuberculin syringe and 23-gauge needle were used for injection, and a pledget of Gelfoam was affixed to the injection site to ensure hemostasis.

Each of the fatty acids was given to a group of five rats. Livers were removed 1,3,5,10, and 15 min after completion of the injection and were then weighed and homogenized in a Waring Blendor with four volumes of Krebs-Ringer bicarbonate buffer. Lipids were extracted by the method of Folch et al. (7). In experiments with octanoic acid, the chloroform-methanol used for extraction contained, as carrier, 2.3 mM octanoic acid and 0.3 mM trioctanoin and was adjusted to pH < 3.0, as previously recommended (4). In experiments with either fatty acid substrate, radioactivity was determined after evaporating the lipid-containing phase to dryness in a rotary evaporator at 40C and redissolving the lipid in hexane. This method yielded recoveries of 88–92% in control albumin solutions containing either ^{14}C octanoic or ^{14}C palmitic acid. Radioactivity in water-soluble products was measured in a dioxane-counting solution (8). In all cases, counting efficiency was determined by a channels ratio method (9).

Experiments in vitro

Immediately after the sacrifice of the rats by decapitation, the liver was removed and sliced with a Stadie-Riggs tissue slicer. Liver slices, weighing 0.4 ± 0.02 g, were incubated in 5 ml of pH 7.4 Krebs-Ringer bicarbonate buffer (½ calcium), containing 5% albumin and 3 μMoles of ^{14}C fatty acid substrate. The buffer mixture was gassed with 5% CO_2-95% oxygen for 30 min prior to the introduction of the liver slices and again just prior to affixing the serum stoppers. Incubations were carried out in 25-ml Erlenmeyer flasks, fitted with center wells, in a Dubnoff shaker at 37C.

To measure the conversion of ^{14}C octanoic or ^{14}C palmitic acid to $^{14}CO_2$, the reaction was stopped by transferring the flask to an ice bath and adding 0.2 ml of 10 N H_2SO_4 to the main compartment and 0.5 ml of hyamine hydroxide (Fisher Chemicals, Inc.

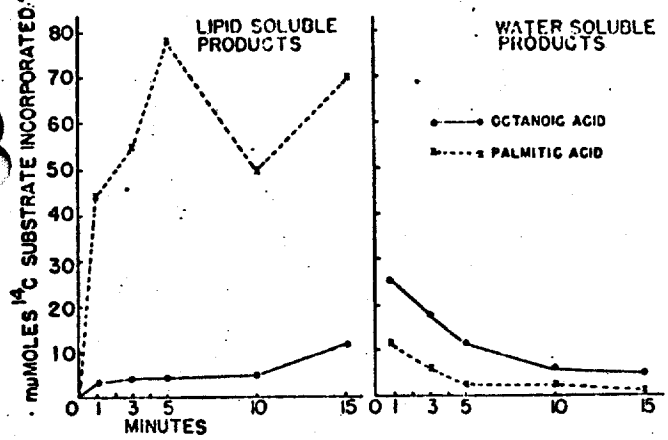

FIG. 1. Incorporation of 1-^{14}C octanoic acid and 1-^{14}C palmitic acid into lipid and water-soluble products of rat liver after intraportal injection.

Watertown, Mass.) to the center well. The flasks were shaken in ice for 30 min, after which the hyamine was transferred to 15 ml of 0.04% Liquifluor in toluene for an assay of radioactivity. Under these conditions $^{14}CO_2$ collection was quantitative, and less than 0.1% of the ^{14}C octanoate was volatilized and taken up by the hyamine. To measure conversion of the fatty acid substrate to lipid and water-soluble products, the reaction was stopped by transferring the flasks to an ice bath, after which the liver slices were removed, washed × 3 in ice-cold distilled water, homogenized with 2.0 ml of Krebs-Ringer bicarbonate buffer at 4C, extracted and assayed for radioactivity, as in the in vivo experiments. In addition, aliquots of the lipid-containing phase were subjected to a) thin-layer chromatography to separate their lipids into classes and to determine the radioactivity in each (10), and b) gas-liquid chromatography (GLC) to analyze the fatty acid composition and isotope content (11). The radioactivity of the carboxyl carbon of the fatty acids was determined, after saponification of the lipid extract, by a modification of the Schmidt reaction (12, 13).

Results

Experiments in vivo

As shown in Figure 1, far less octanoic acid than palmitic acid was incorporated into hepatic lipids

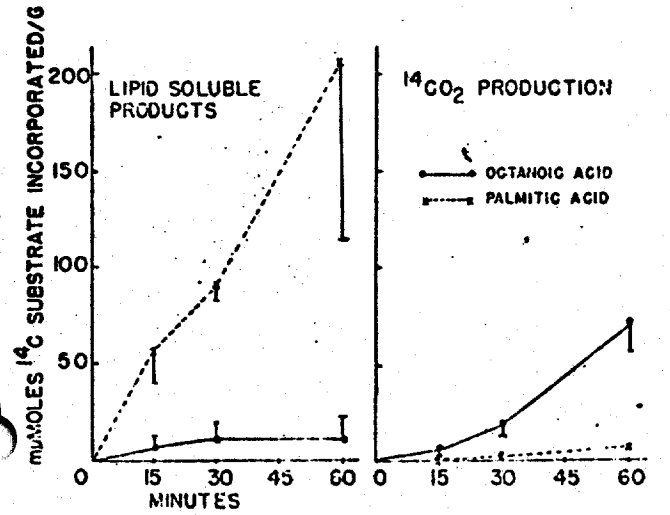

FIG. 2. Incorporation of 1-^{14}C octanoic and 1-^{14}C palmitic acid into lipid-soluble products and $^{14}CO_2$ by rat liver slices. Each point represents the mean ± 1 S.D. of three experiments.

TABLE I

Metabolism of 1-^{14}C Octanoic and 1-^{14}C Palmitic Acid to $^{14}CO_2$ and Lipid-Soluble Products by Liver Slices[1]

Substrate	$^{14}CO_2$	Lipid-soluble products
(3 μMoles)	(mμMoles ^{14}C fatty acid incorporated/g liver slice)	
Octanoic acid	213.7 ± 26.3	14.0 ± 4.3
Palmitic acid	15.0 ± 2.5	307.0 ± 15.0
p value	<0.01 (n = 3)	<0.001 (n = 6)

[1] Incubations were for 2 hr. Results expressed as mean ± SEM.

after the injection of these fatty acids into the portal vein. During the 15-min period of observation the ratio of octanoic:palmitic acid incorporation ranged between 1:6 and 1:18; the difference between the two was evident as early as 1 min after the injection. The amount of labeled lipid in the liver after injection of either fatty acid tended to increase with time, suggesting that, under the conditions of this experiment, repeated passages through the liver were required for extraction of the fatty acids from the plasma.

In contrast to its slower rate of incorporation into hepatic lipids, octanoic acid yielded two to four times more water-soluble material than palmitic acid. After injection of either fatty acid the concentration of labeled water-products in the liver was maximal at 1 min; then fell progressively (Figure 1).

Experiments in vitro

When liver slices were incubated with fatty acids, significantly less octanoic acid than palmitic acid was incorporated into hepatic lipids, as in the in vivo experiments (Figure 2). In contrast, the oxidation of octanoic acid to CO_2 by liver slices was significantly greater than that of palmitic acid. As shown in Table I, a high proportion of the labeled octanoic acid taken up by liver slices during a 2-hr incubation period was oxidized to CO_2, and very little was incorporated into lipid-soluble products. The converse was true of palmitic acid.

The lipid-soluble products formed from octanoic and palmitic acid in this experiment were characterized by separating the lipid classes by TLC (Table II). Triglycerides accounted for approximately half the radioactivity incorporated into the lipids of incubated liver slices. However the other lipid products of these two fatty acids differed in that octanoic acid yielded a higher proportion of unesterified fatty acids and a lower proportion of phospholipids and monoglycerides than did palmitic acid.

The lipid-soluble products were further characterized by GLC of the fatty acid methyl esters which were formed by transmethylation of the lipid extract. This revealed that fatty acids accounted for 92.8 ± 2.0% of the lipid radioactivity in the octanoate experiments and for 96.0 ± 0.4% in the palmitate experiment, indicating that little conversion of these

TABLE II

Lipid-Soluble Products Formed from 1-^{14}C Octanoic and 1-^{14}C Palmitic Acid by Liver Slices[1]

^{14}C Substrate (3 μMoles)	Percentage distribution of total lipid radioactivity		
	Phospholipids and monoglycerides	Unesterified fatty acids	Triglycerides
Octanoic acid (6)	10.3 ± 2.4	38.2 ± 6.9	48.3 ± 4.6
Palmitic acid (6)	31.7 ± 1.7	17.0 ± 1.8	46.3 ± 5.6
p value	<0.001	<0.05	NS

[1] Lipid soluble products migrating with diglycerides, cholesterol, and cholesterol esters comprised 5.2% ± 1.2 of the lipid soluble products from octanoic acid and 5.0% ± 0.3 from palmitic acid. Numbers in parentheses refer to the number of experiments. The same six rat livers were used for these data as were employed for the lipid products data in Table I. Incubations were for 2 hr. Results expressed as mean ± SEM.

TABLE III

Distribution of ^{14}C Among Fatty Acids After Incubating Liver Slices with 1-^{14}C Octanoic or 1-^{14}C Palmitic acid[1]

Substrate (3 μMoles)	Fatty acid product					Fatty acid label in carboxyl groups
	<16:0[2]	16:0	16:1	18:0	18:1, 18:3 20:0, 20:4	
	(% Total fatty acid label in each)					(%)
Octanoic acid	17.5 ± 6.4	42.2 ± 8.6	12.5 ± 2.2	4.8 ± 0.5	23.0 ± 5.1	80.1 ± 2.4
Palmitic acid	1.8 ± 0.6	77.4 ± 2.9	12.0 ± 1.7	4.0 ± 0.8	4.8 ± 0.7	96.8 ± 1.1
p value	<0.05	<0.01	NS	NS	<0.05	<0.001

[1] The same six rat livers were used for these data as were employed for the data in Table II. Incubations were for 2 hr. Results expressed as mean ± SEM.

[2] <16:0 refers to fatty acids the carbon chain-lengths of which are less than 16 but greater than six.

substrates to glyceride glycerol, phospholipid bases, or cholesterol had occurred. From the data in Table III it can be seen that, after 2 hr of incubation, less than 10% of the label in ^{14}C palmitic acid was found in hepatic fatty acids of longer chain-length. In contrast, most of the ^{14}C octanoic acid underwent elongation. Of particular note, less than 2% of the fatty acid radioactivity after incubation was recovered in the octanoate fraction. Moreover a large proportion of the label (23%) was found in C18 and C20 unsaturated fatty acids. Since 80% of the ^{14}C in these longer-chain fatty acids which were derived from 1-^{14}C octanoic acid was present in carboxyl groups (Table III), it is highly probable that the octanoate was oxidized to acetate prior to its incorporation into long-chain fatty acids.

Discussion

From the data presented it is evident that, compared with palmitic acid, octanoic acid is a poor substrate for lipid synthesis in the liver but is more readily catabolized to CO_2 and water-soluble products. Similar differences in the metabolism of these two fatty acids have been observed in intestinal slices (5). However their behavior in the liver differs from that in the intestine in several respects. Whereas, in the liver, the bulk of the octanoic acid that is not catabolized to CO_2 and water-soluble products is converted to long-chain fatty acids, little of the octanoic acid that enters the intestinal mucosa is elongated; most of the uncatabolized fraction was recoverable intact and unesterified. Elongation of palmitic acid occurs to a small but significant degree in the liver but almost not at all in the intestinal mucosa. In the liver, comparable fractions of fatty acids derived from octanoic and palmitic acid are incorporated into triglycerides whereas, in the intestine, there is preferential incorporation of fatty acids derived from palmitic acid but not octanoic acid.

Previous studies have shown that excluding the liver from the circulation does not significantly reduce the amount of $^{14}CO_2$ recovered in the expired air of animals given 1-^{14}C octanoic acid (20). This finding suggests that octanoic acid may be preferentially oxidized to CO_2 rather than incorporated into other lipids not only in the liver and intestine but also in other extrahepatic tissues. It is quite possible therefore that octanoic acid, in supporting growth in man (15) and animals (16,17), merely spares carbohydrate and fat by providing caloric energy.

ACKNOWLEDGMENTS

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5. HEPATIC METABOLISM OF MEDIUM CHAIN FATTY ACIDS

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TRIGLYCERIDES CONTAINING MEDIUM CHAIN LENGTH FATTY ACIDS differ markedly from those containing long chain fatty acids with respect to their intestinal absorption and intramucosal metabolism, as has been discussed. Long chain triglycerides undergo intraluminal hydrolysis to monoglycerides, fatty acids, and glycerol, are absorbed, and these products are recombined within the intestinal mucosa to form triglycerides, and are then exported as chylomicra via the lymphatics. In contrast, medium chain triglycerides, which contain principally octanoic acid, can be absorbed without prior hydrolysis although intramucosal lipolysis occurs. Medium chain fatty acids released by the action of an intramucosal lipase are transported to the liver via the portal vein, as shown by Bloom, Chaikoff and Reinhardt (1951), Kiyasu, Bloom, and Chaikoff (1952), and by Borgström (1955).

The possibility that medium chain and long chain triglycerides also differ with respect to their metabolism in other tissues has not been examined previously. Since medium chain triglycerides are being used therapeutically, with apparent good effect, in the management of steatorrhea due to a variety of causes, the metabolic fate of such lipid is of considerable interest and possible importance. The data to be presented, based on *in vitro* and *in vivo* studies in the rat, show that in all tissues examined, including liver, kidney, heart, striated muscle, brain and adipose tissue, octanoic acid is much more readily oxidized to CO_2, but less effectively incorporated into tissue lipids, than palmitic acid.

In vivo studies were performed by injecting into the portal vein either octanoate-C^{14} or palmitate-C^{14} as an albumin complex, extracting the hepatic lipid and measuring its radioactivity. As can be seen in Fig. 5-1,

Cap. acid

LIPID FORMATION IN VIVO FROM MEDIUM & LONG CHAIN FATTY ACIDS BY RAT LIVER

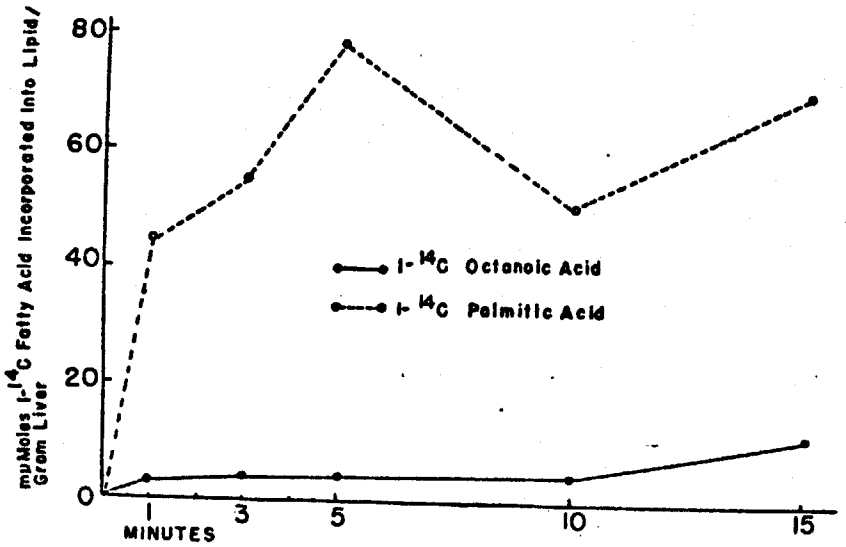

Fig. 5-1. Male rats weighing 180-220 g were anesthetized with Nembutal (pentobarbital sodium) and the portal vein was isolated through a midline incision. One ml of a solution containing 1.2 μ moles (2.4 μc) of either octanoate-1-C^{14} or palmitate-1-C^{14} complexed by homogenization with 10% Janel fatty acid-poor bovine albumin in pH 7.4 Krebs-Ringer bicarbonate buffer was injected into the portal vein in 1.0 minute with a 1 ml tuberculin syringe and a 23-gauge needle. The livers were removed 1,3,5,10, and 15 minutes after injection, weighed, and homogenized in a Waring Blender with 4 volumes of Krebs-Ringer bicarbonate buffer. The lipids were extracted with chloroform–methanol (Folch *et al.*, 1957), modified for medium chain fatty acid recovery (Playoust and Isselbacher, 1964). Isotope content was determined by liquid scintillation spectrometry after evaporating the lipid-containing phase to dryness in a rotary evaporator at 40 C, redissolving the lipid in hexane, and transferring an aliquot to a 0.04% solution of Liquiflour in toluene.

far less octanoic acid than palmitic acid was incorporated into hepatic lipid. The difference was apparent as early as one minute following injection and persisted over the 15-minute period of observation. At no time did the amount of octanoate incorporated into lipid soluble products exceed one-sixth that of palmitate.

As is evident from Fig. 5-2, the results obtained from *in vitro* studies confirmed the *in vivo* observations. Liver slices were prepared from 180-220 g male rats and incubated with the carboxyl-labeled fatty acid in a buffer containing albumin. Again, the rate of incorporation of octanoic acid into lipid soluble products was much less than that of palmitic acid. In contrast to its slower rate of incorporation into hepatic lipids, octanoate was much more readily oxidized to carbon dioxide than was palmitic acid.

LIPID & $^{14}CO_2$ FORMATION FROM MEDIUM & LONG CHAIN FATTY ACIDS BY RAT LIVER SLICES

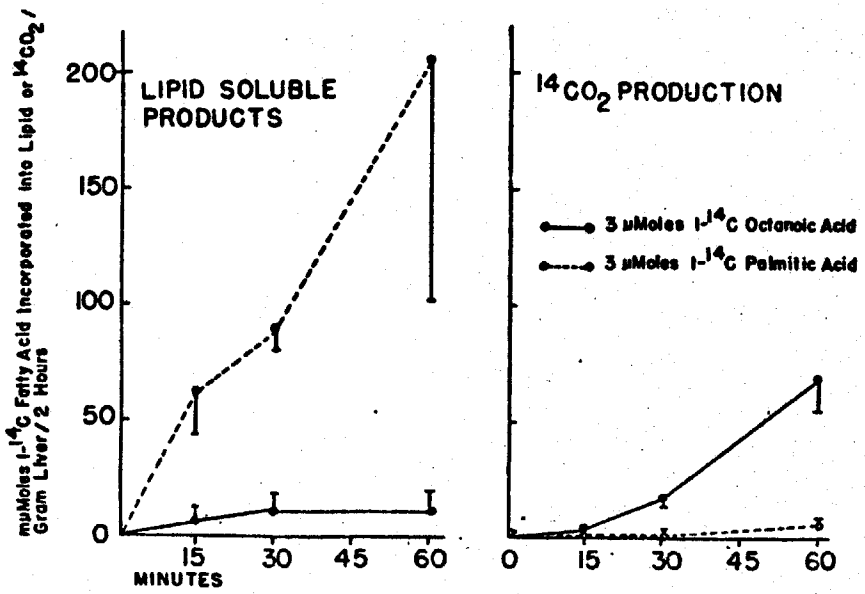

Fig. 5-2. Liver slices prepared with a Stadie-Riggs tissue slicer and weighing 0.4± 0.02 g were incubated in 5 ml of Krebs-Ringer bicarbonate (½ calcium) containing 5% albumin and 3 μ moles of the C^{14} fatty acid substrate. The buffer mixture had been gassed with 5% CO_2/95%O_2 prior to the introduction of the slices and again before stoppering with serum caps. The incubations were in a Dubnoff shaker at 37 C in 25-ml Erlenmeyer flasks fitted with center wells. $C^{14}O_2$ production was determined by liquid scintillation spectrometry after transferring flasks to an ice bath, adding 0.2 ml H_2SO_4 to the main compartment, 0.5 ml Hyamine hydroxide to the center well, shaking in ice for 30 minutes and transferring the Hyamine to 15 ml of 0.04% Liquiflour in toluene. Lipid-soluble product formation was determined in separate flasks as in the *in vivo* experiments described in Fig. 5-1 after the slices had been removed, washed three times in ice cold distilled water, homogenized with 2.0 ml Krebs-Ringer bicarbonate buffer at 4 C, and the lipids extracted. Each point represents the mean ± 1 SD of three experiments.

Because the previous nutritional state of the animal is known to modify lipid metabolism (Lossow and Chaikoff, 1955), the oxidation of medium and long chain fatty acids and their incorporation into lipid were measured in liver slices from male rats either fed or fasted for 17 hours prior to sacrifice. As can be seen from Table I, fasting depressed both the oxidation of octanoic acid to $C^{14}O_2$ and its incorporation into hepatic lipid. Nevertheless, the amount of octanoic acid oxidized was still substantial. In contrast, fasting decreased the oxidation of palmitate to a greater extent than octanoate but had no effect on the incorporation of palmitate into hepatic lipid.

The data in Table II show that the sex of the rat from which the liver was derived did not alter either the oxidation of octanoic acid or its incorporation into lipid.

Table I

Metabolism of Medium and Long Chain Fatty Acids to CO_2 and Lipid Soluble Products by Rat Liver Slices[a]

I. EFFECT OF PREVIOUS DIET

Dietary Regimen	Substrate (3 μmoles)	$C^{14}O_2$ Production	Lipid soluble products
		mμmoles fatty acid-1-C^{14} incorporated per gram liver per 2 hours	
Fed	Octanoic acid-1-C^{14}	255 ± 87	8 ± 2
Fasted	Octanoic acid-1-C^{14}	127 ± 20	2 ± 0
		$P<0.001$	$P<0.001$
Fed	Palmitic acid-1-C^{14}	26 ± 7	232 ± 18
Fasted	Palmitic acid-1-C^{14}	7 ± 1	239 ± 14
		$P<0.01$	NS[b]

[a] Methodology was identical to that used in the experiments in Fig. 5-2 except that all incubations were for 2 hours. Liver slices were prepared from 7-week-old male rats weighing on the average 210 g which had been either fed or fasted 17 hours before sacrifice. Each number represents the mean ±1 SD of six animals and each liver provided slices to assay either $C^{14}O_2$ or lipid production from both fatty acid substrates. The statistical significance of differences between groups was calculated by the paired "t" test (Mainland, 1963).
[b] NS = not significant.

Table II

Metabolism of Medium and Long Chain Fatty Acids to CO_2 and Lipid Soluble Products by Rat Liver Slices[a]

II. EFFECT OF SEX OF DONOR

Sex	Substrate (3 μmoles)	$C^{14}O_2$ Production	Lipid soluble products
		mμmoles fatty acid-1-C^{14} incorporated per gram liver per 2 hours	
Male	Octanoic acid-1-C^{14}	85 ± 46	5 ± 2
Female	Octanoic acid-1-C^{14}	104 ± 44	6 ± 3
		NS[b]	NS
Male	Palmitic acid-1-C^{14}	8 ± 4	247 ± 2
Female	Palmitic acid-1-C^{14}	10 ± 2	304 ± 27
		NS	$P<0.05$

[a] Methodology was identical to that used for the data in Table I. All rats were fed until sacrifice. They were 7 weeks old with an average weight of 215 g for the males and 195 g for the females.
[b] NS = not significant.

While the age of the animal apparently did not influence the metabolism of palmitate by the liver, there seemed to be some decrease in the oxidation of octanoic with advancing age (Table III).

Table III

Metabolism of Medium and Long Chain Fatty Acids to CO_2 and Lipid Soluble Products by Rat Liver Slices[a]

III. EFFECT OF AGE OF DONOR

Age (weeks)	Substrate (3 μmoles)	$C^{14}O_2$ Production	Lipid soluble products
		mμmoles fatty acid-1-C^{14} incorporated per gram liver per 2 hours	
4½	Octanoic acid-1-C^{14}	173 ± 40	3 ± 3
11	Octanoic acid-1-C^{14}	117 ± 37	2 ± 2
		$P<0.05$	NS[b]
4½	Palmitic acid-1-C^{14}	11 ± 3	222 ± 30
11	Palmitic acid-1-C^{14}	8 ± 4	204 ± 27
		NS	NS

[a] Methodology as in Table I. Male rats fed until sacrifice were the donors of the liver slices and the average weight of the 4½-week-old rats was 110 g and of the 11-week-old rats 335 g.
[b] NS = not significant.

It might be noted that in all the preceding experiments standard deviations were rather wide, indicating either variability in preparing tissue slices or more probably, variability from animal to animal. Nevertheless, the differences in the metabolism of octanoic and of palmitic acid were quite consistent and highly significant under all experimental conditions.

In order further to explore possible differences between the metabolism of medium and long chain fatty acids, their lipid products in the livers were characterized (Table IV). The lipid classes were separated by thin layer

Table IV

Lipid Soluble Products Formed from Medium and Long Chain Fatty Acids by Rat Liver

Substrate (3 μmoles)	Per cent distribution of total lipid radioactivity		
	Triglycerides	Unesterified fatty acids	Phospholipids and monoglycerides
Octanoic acid-1-C^{14}	48 ± 5	38 ± 7	10 ± 2
Palmitic acid-1-C^{14}	46 ± 6	17 ± 2	32 ± 2
	NS[b]	$P<0.05$	$P<0.001$

[a] Liver slices from six fed male rats (average weight 200 g) were incubated for 2 hours and the lipids were extracted. The methods used are described in Table I. Of the octanoic acid 14.0 ± 4.3 and of the palmitic acid 307.0 ± 15.0 mμmoles was incorporated into lipid per gram liver ($P<0.001$). Aliquots of the lipid extract were subjected to thin layer chromatography to separate the lipids into classes and to determine the radioactivity in each.
[b] NS = Not significant.

chromatography and their isotope content determined (Scheig and Isselbacher, 1965). With both C^{14} fatty acids, approximately half the radioactivity recovered in lipid was found in triglyceride. However, the other lipid products of these two fatty acids differed significantly. Thus, 38% of the lipid radioactivity from octanoate but only 17% of that from palmitate was in the form of unesterified fatty acids. In contrast, only 10% of the octanoic acid but 32% of the palmitate was incorporated into monoglycerides and phospholipids.

The fatty acid constituents of these lipid products were determined by preparing the fatty acid methyl esters and assaying the isotope content of each after gas-liquid chromatographic separation (Scheig, Alexander, and Klatskin, 1966). Interestingly, when octanoic and palmitic acids served as substrates, 93% and 96%, respectively, of the lipid radioactivity was recovered in the fatty acid moieties of the complex lipids, indicating that neither substrate had been converted to glyceride glycerol, phospholipid bases or cholesterol to any appreciable extent. As is evident from Table V,

Table V

Distribution of C^{14} amongst Fatty Acids after Incubating Liver Slices with Octanoate-1-C^{14} or Palmitate-1-C^{14}[a]

Substrate (3 μmoles)	(Per cent total fatty acid label in each)				
	<16.0[b]	16:0	15:1	18:0	18: and 20: unsaturated
Octanoic acid-1-C^{14}	17 ± 6	42 ± 9	13 ± 2	5 ± 1	23 ± 5
Palmitic acid-1-C^{14}	2 ± 1 $P<0.05$	77 ± 3 $P<0.01$	12 ± 2 NS[c]	4 ± 1 NS	5 ± 1 $P<0.05$

[a] The same six rat livers and the same methods were used for this data as were employed for the data in Table IV. Aliquots of the lipid extract were subjected to gas-liquid chromatography to analyze fatty acid composition and isotope content.
[b] <16:0 refers to fatty acids whose carbon chain lengths are less than 16 but greater than 6.
[c] NS = Not significant.

most of the palmitate was incorporated into tissue lipid unaltered, only 9% having been elongated and 12% desaturated. In contrast, the bulk of the octanoate incorporated into liver lipids was in elongated fatty acids, only 17% not having been converted to long chain fatty acids. Actually, less than 2% of the recovered lipid radioactivity remained in the form of octanoate. Moreover, a rather large proportion of the octanoate, namely 36%, was in fatty acids which were both elongated and desaturated, and the radioactivity was contained in 16, 18, and 20-carbon unsaturated fatty acids. Octanoate probably had been oxidized to acetate or acetyl coenzyme

A prior to being incorporated into long chain fatty acids either by synthesis via the malonyl-CoA pathway or, more likely, by 2-carbon chain addition to endogenous fatty acids in mitochondria (Harlan and Wakil, 1962; 1963). In support of the latter hypothesis, it was found by Schmidt degradation that 80% of the label in the fatty acids derived from octanoate was contained in carboxyl groups (Phares, 1951; Brady, Bradley, and Trams, 1962).

That medium chain fatty acids are more readily oxidized than long chain fatty acids was evident not only in liver and in the intestine (Greenberger, Franks, and Isselbacher, 1965), but also in other tissues. Thus, as shown in Fig. 5-3, the oxidation of octanoate was significantly greater than

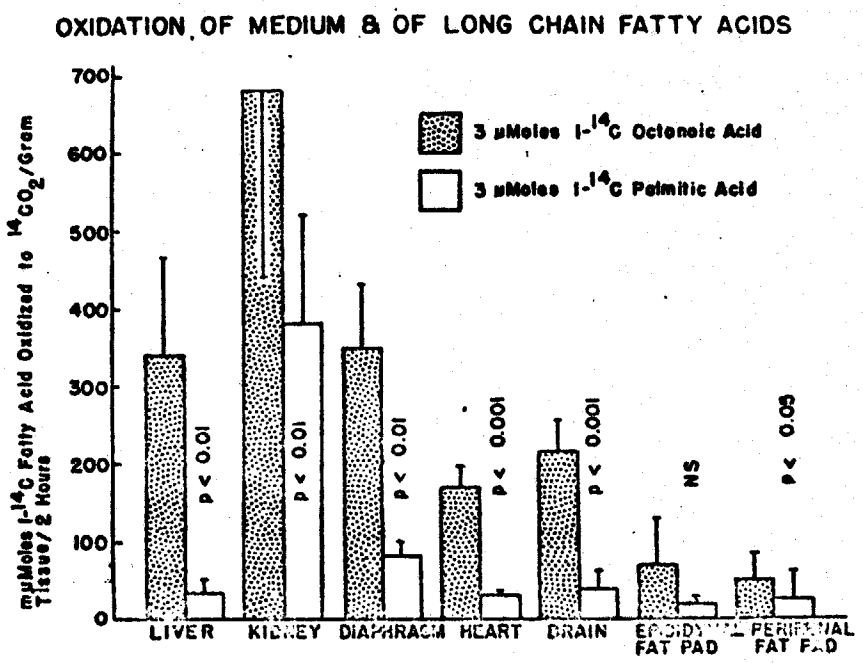

Fig. 5-3. Methodology was the same as that used for the data in Table I. Male rats, weighing on the average 250 g, were fed until sacrifice. One animal provided all the tissues. Each bar represents mean ± 1 SD of determinations on six animals. The average time from sacrifice until start of incubations was 40 minutes. The slices in each flask weighed about 100 mg except for brain which averaged 50 mg, and adipose tissue which averaged 150 mg.

palmitate in kidney, diaphragm, heart, brain, and adipose tissue. Noteworthy is the magnitude of the oxidation observed in all these issues. In contrast to its extensive oxidation, the data in Fig. 5-4 show that little of

LIPID SYNTHESIS FROM MEDIUM & LONG CHAIN FATTY ACIDS

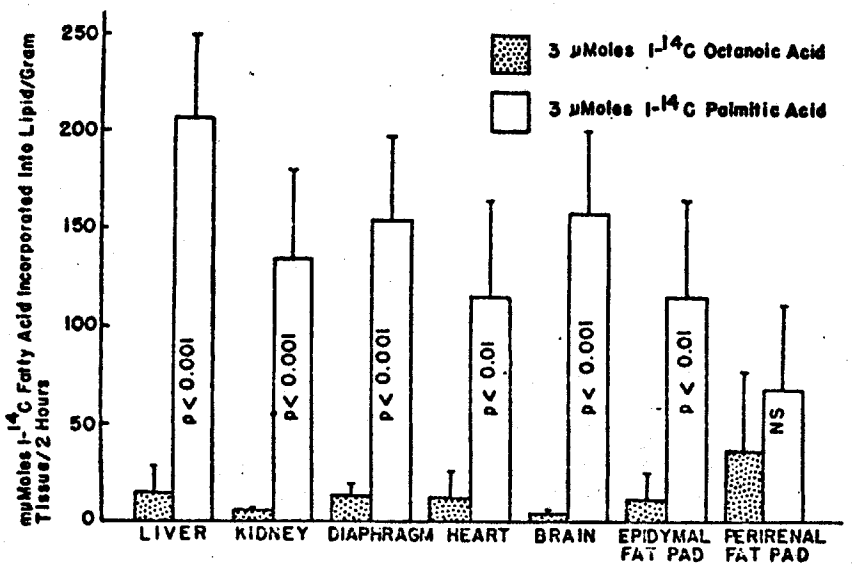

Fig. 5-4. See Fig. 5-3 and Table I methodology, text for discussion.

the octanoate was used for tissue lipid synthesis, especially when compared to tissue utilization of palmitate.

From the data presented, it is evident that, compared to long chain fatty acids, medium chain fatty acids are poorly utilized for tissue lipid synthesis but are readily catabolized to CO_2. In comparing our data on the liver with those of Greenberger, Franks and Isselbacher (1965) on the intestine, it is apparent that the type of lipid formed from octanoic acid differs in these two tissues. Thus, in the liver the bulk of the octanoic acid that is not catabolized is converted to long chain fatty acids which are esterified, while in the intestine little is used for elongation or esterification.

In summary, the metabolism of a representative medium and a long chain fatty acid was studied in the rat. Octanoic acid proved to be a relatively poor substrate for lipid synthesis compared with palmitic acid, but was more readily oxidized to CO_2. These data suggest that triglycerides containing octanoic acid may support growth by providing calories, thereby sparing carbohydrate and other fats from catabolism. Accordingly, the therapeutic use of medium chain triglycerides as a more readily absorbed dietary lipid in patients with malabsorption due to biliary tract, pancreatic, or intestinal disease would appear to have a rational basis.

Acknowledgments

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Questions

Chairman Senior: From your data, where you analyzed the liver fatty acids after octanoate metabolism, you indicated that a fairly large fraction of the recovered radioactivity was in longer fatty acids, indicating elongation. Is the mechanism of this elongation a preliminary breakdown to acetate and then a resynthesis of fatty acids *de novo* (in which case the label would be distributed throughout the chain), a mitochondrial addition of labeled acetate to fatty acids which would place the label in the C-1 position, or is it a direct elongation of the octanoate by other 2-carbon additions? Do you have any evidence for direct elongation of the octanoate, in which case the C-1 label would be found in C-9 or C-11 position?

Dr. Scheig: As you know, fatty acid synthesis can occur either *de novo* from 2-carbon fragments by the malonyl-CoA pathway in the cell sap or by an elongation reaction, in which a 2-carbon fragment is added to a preexisting fatty acid. This latter occurs principally in the mitochondria and perhaps somewhat in the microsomes. There are two lines of evidence that help me to distinguish which synthetic process is being used during octanoate metabolism: First, when the fatty acids recovered after the two-hour incubation were decarboxylated, that is the terminal CO_2 group was removed and counted, it was found by Schmidt degradation that 80% of the fatty acid radioactivity was in that carboxyl group (Phares, 1951; Brady, Bradley, and Trams, 1962); second, when the fatty acid products which result from incubation of a liver slice with acetate-1-C^{14} or following the injection of acetate-1-C^{14} into the portal vein were studied, we found that the fatty acid products were quite similar to those observed when octanoic acid was the substrate. So I think both these sets of findings suggest that the octanoic acid had been oxidized to 2-carbon fragments and then added to a preexisting fatty acid, that is, the octanoic acid carbon is used in an elongation reaction.

Chairman Senior: Your opinion, then, is that the octanoate is catabolized to acetate first, almost exclusively, rather than being itself elongated.

Dr. Scheig: Right.

Dr. Hans Kaunitz (Columbia University, New York): I wanted to ask whether you noted that the linoleate content of the diet that you provided for your animals had any bearing on your results, because we have found repeatedly that the amount of linoleate in the diet has a strong bearing on the incorporation of medium chain triglycerides into tissue triglycerides. We found that a linoleate-free diet leads to no incorporation, but if you

increase linoleate in the diet up to 10–15%, the fatty acids from medium chain triglycerides will be incorporated into the liver triglycerides or tissue triglycerides.

Dr. Scheig: The diet used was Purina lab chow, which is 3.8% lipid, of which about a third is linoleate (Scheig, Alexander and Klatskin, 1966). I don't know how many grams of chow a day these animals were eating, so I couldn't calculate how much linoleate they were actually getting.

Dr. G. Steiner: In your studies comparing the metabolism of octanoate relative to palmitate you have shown decreased recovery of label in lipid and increased CO_2 production. This raises the possibility of increased lipid turnover and hence decreased recovery of labeled lipid because it has just been oxidized to CO_2. I would be interested to know what controls you have done to exclude this possibility.

Dr. Scheig: I'm not sure what kind of controls you would use to exclude it.

Dr. Steiner: Have you any data on the specific activity of the label in the lipids recovered? Have you any kinetic data on what is happening in this situation?

Dr. Scheig: No.

Dr. Peter R. Holt: Dr. Senior's comment about Robert Frost at the beginning of the symposium might well apply to medium chain fatty acids with respect to the diversion between esterifying pathway and oxidation. You have mentioned a number of tissues in your presentation. One tissue apparently differs and that is the mammary tissue, in which there is significant esterification of octanoate. It is intriguing, I think, and important in this type of symposium to speculate upon the reasons why these differences occur. Are these due only to enzyme specificities or could there be other mechanisms which will divert this particular type of lipid into these pathways?

Another point I would like to make is the possibility of the interrelationship of esterification with glucose metabolism. We have found in studies on intestinal mucosa that if you starve the animal until glycogen disappears and then attempt to show esterification with long chain lipid, it in fact decreases, while if in your incubation medium you add glucose, it increases. The intriguing point is that when one adds MCT, instead of glucose, one would still be able to show an increased esterification. This in fact is partly due to glycerol. Would you comment on that, too?

Dr. Scheig: I really have nothing to say on the latter point. On the former point we have no data. I think you could speculate that this might be due to different enzyme specificity. One could also speculate, as Dr. Hofmann mentioned earlier, that this had to do with different solubilities of the substrate. In order to compare these as closely as possible, both oc[illegible] and palmitate were complexed with albumin. But perhaps the release of the octanoate from the albumin complex is more readily accomplished than is the release of palmitate. Tissue slices were used, and so the transport across the cell membrane still had to take place before either oxidation or incorporation into various lipid products could occur. I think there is a multiplicity of factors and we have no data to determine which of these factors are involved.

Dr. Daniel Rudman: I wanted to bring out a question you just touched on, Dr. Scheig. Spector and Steinberg (1965), in studying the utilization of fatty acids by tumor cells, showed that the primary determinant was the concentration of unbound fatty acid in the medium rather than the total quantity present. Undoubtedly with equivalent amounts of albumin and equal quantities of octanoate and palmitate your concentration of free octanoate must have been much greater than the concentration of free palmitate, so that before one could accept the concept that the medium chain fatty acid is much more readily utilized one would want to study the problem of equivalent concentrations of unbound fatty acid.

J. Appl Physiol. 19 (2): 335-337 (1964)

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Octanoic acid absorption and oxidation in humans[1]

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SCHWABE, ARTHUR D., LESLIE R. BENNETT, AND LANCE P. BOWMAN. *Octanoic acid absorption and oxidation in humans.* J. Appl. Physiol. 19(2): 335-337. 1964.—The absorption and oxidation of carboxyl-labeled octanoic acid as determined by monitoring of expired $C^{14}O_2$ was studied in 27 subjects. In 12 normal volunteers there was no significant difference in the recovery of $C^{14}O_2$ after oral and intravenous administration of the isotopically labeled octanoic acid. Administration of 100 g [illegible] mouth did not seem to inhibit octanoic acid oxidation [illegible] subjects. Infusion of 1 g unlabeled octanoate also did not significantly affect the oxidation of radioactive octanoate in five subjects. These observations in humans tend to confirm animal studies which have shown that octanoic acid is well absorbed from the gastrointestinal tract and rapidly oxidized. The oxidation of small amounts of this fatty acid appears to proceed at the same rate despite moderate blood levels of glucose or octanoic acid. These characteristics of short chain fatty acid absorption and oxidation can be exploited in the study of fat absorption in human subjects.

intestinal absorption fatty acid metabolism

OCTANOIC ACID, an 8-carbon fatty acid found in esterified [illegible] in coconuts, butter, and certain other dietary fats, has not been extensively used in clinical medicine. Animal studies have shown that octanoic acid is rapidly absorbed from the intestine, carried to the liver via the portal venous system, and rapidly and completely oxidized (6). It has also been observed in rats that the oxidation of carboxyl-labeled octanoic acid is relatively uninhibited by the administration of carbohydrates [illegible]. These physiological properties of octanoic acid first suggested its use and that of its ester, glyceryl trioctanoate, in the study of intestinal absorption in man (8). The present study was undertaken to determine the degree of octanoate absorption and its oxidation under various conditions in human subjects.

METHODS

The water-soluble octanoic acid labeled with C^{14} in the carboxyl position, henceforth referred to as RAO (radioactive octanoate) for brevity, is prepared by the New England Nuclear Corporation with a specific activity of 1.99 mc/mmole. The isotope was administered in 2- to 3-μc doses in the fasting state to a group of normal volunteers and to patients without evidence of pulmonary, liver, or heart disease, or abnormal lipid metabolism. On every occasion following the administration of the isotope, each subject was immediately placed under a plastic helmet and the expired $C^{14}O_2$ was continuously monitored and recorded by a method outlined in a previous paper (8) (Fig. 1). The cumulative expired radioactive CO_2 recovered in a 50-min period was expressed as the percentage of the administered dose. The collection of expired $C^{14}O_2$ was terminated after 50 min because these and previous experiments have shown that peak recovery of $C^{14}O_2$ occurred invariably between 15 and 30 min. Thereafter $C^{14}O_2$ recovery was falling off steadily. Lossow and Chaikoff (7) have shown that the $C^{14}O_2$ recoveries in fed and fasted rats after 6 hr were 82 and 90%, respectively, following intravenous injection of octanoic acid. The hourly recovery of $C^{14}O_2$ was greatest during the first hour of testing.

The subjects were divided into three experimental groups, all of which received one or more intravenous injections of RAO to establish control recovery values of $C^{14}O_2$.

The first experiment, involving a group of 12 healthy medical students, was designed to compare the recovery of $C^{14}O_2$ after orally and intravenously administered RAO. Each subject received 2 or 3 μc of the labeled compound orally and intravenously on separate occasions at least 3 days apart. Monitoring of expired $C^{14}O_2$ was performed after each test dose (Table 1).

The second study performed on ten subjects (four normal adults and six patients) was designed to determine the effect of

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Cap. Acid

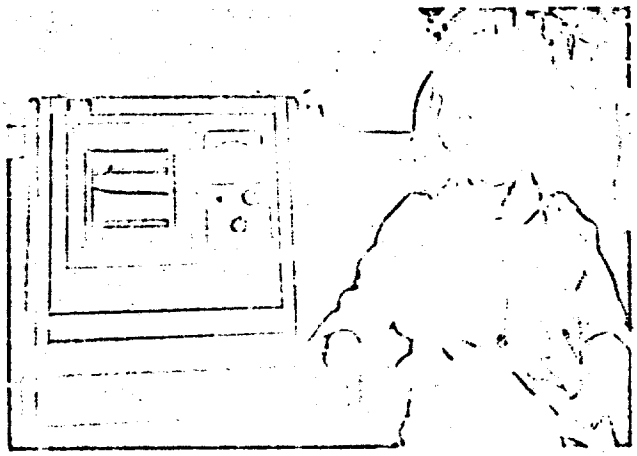

FIG. 1. Apparatus for measurement of expired radioactive carbon dioxide.

glucose administration on octanoic acid oxidation. Initially each of the ten subjects received one or more intravenous control injections of 2 μc RAO at least 3 days apart. Subsequently, again at least 3 days following the last test, each one received 100 g glucose dissolved in 250 ml lemon-flavored tap water. Twenty minutes after the glucose had been ingested each individual was placed under the helmet and given 2 μc of RAO intravenously. Monitoring of expired $C^{14}O_2$ was carried out after each test dose (Table 2).

In the third group of five patients the effect of fatty acid load on RAO oxidation was investigated. Three or more days after the base-line determination, each subject received an intravenous infusion of 1 g unlabeled octanoic acid dissolved in 250 ml 1.3% sodium bicarbonate at the rate of 60 drops/min. At the end of the infusion 2 μc RAO were again administered intravenously. The amounts of $C^{14}O_2$ recovered after each test dose are compared on Table 3.

TABLE 1. *Comparison of $C^{14}O_2$ recovery after oral and intravenous RAO administration in twelve normal males*

Subj.	A. Intravenous RAO			B. Oral RAO			A − B % Diff.
	Dose, μc	DT, min	% Recovered	Dose, μc	DT, min	% Recovered	
JM	2	2	16.5	2	4	15.7	+0.8
EM	2	1	14.9	2	4	17.7	−2.8
JH	2	2	15.1	2	3	16.3	−1.2
DY	3	2	15.2	3	3	16.1	−0.9
HB	3	2	17.5	3	3	18.1	−0.6
RP	3	1	16.7	3	4	16.5	+0.2
PA	2	1	12.7	2	3	16.3	−3.6
PM	2	2	15.3	2	4	14.8	+0.5
BD	2	2	18.2	2	5	13.9	+4.3
RA	2	2	19.0	2	5	13.0	+6.0
LB	2	2	12.0	3	6	11.0	+1.0
MW	3	2	15.1	3	4	15.5	−0.4
Avg.			15.7			15.4	

The average significance of comparison = $P > 0.7$.

RESULTS

In the first group of 12 medical students no statistically significant difference in the recovery of $C^{14}O_2$ after intravenous (column A) and oral (column B) administration of RAO was noted (Table 1). The delay time (DT), i.e., the interval from the point at which RAO was administered to the point at which activity was first observed on the recorder chart, ranged from 1 to 2 min for the intravenously administered and from 3 to 6 min for the orally administered isotope.

In the second group, 18 control and 10 postglucose intravenous RAO tests were performed in the ten subjects. No statistically significant differences of recovered $C^{14}O_2$ before and after glucose administration were noted, suggesting that glucose ingestion did not inhibit octanoic acid oxidation (Table 2).

The results of five control (column A) and five postoctanoic

TABLE 2. *Effect of glucose on RAO oxidation*

Subj.	A. Intravenous RAO Control			B. Intravenous RAO, After 100 g Glucose			A − B % Diff.
	Dose, μc	DT, min	% Dose recovered	Dose, μc	DT, min	% Dose recovered	
NB	2	2	21.9	2	2	24.5	−2.6
BR	2	2	24.0	2	2	31.4	−7.4
AS	2	3	24.5	2	1	24.9	−.4
JH	2	2	25.9	2	1	21.0	+4.9
MB	2	2	14.3	2	2	21.7	−7.5
	2	2	14.1				
SH	2	2	16.9	2	2	15.7	+.7
	2	2	15.9				
DP	2	2	20.0	2	1	20.5	+.3
	2	2	21.6				
BJ	2	2	18.1	2	2	21.0	−2.1
	2	1	18.3				
	2	1	20.4				
AM	2	3	17.2	2	2	20.2	−3.9
	2	2	14.5				
	2	2	17.4				
LA	2	1	19.0	2	2	19.7	−2.3
	2	1	15.7				
Avg.			18.9			22.0	

The average significance of comparison = $P > 0.2$.

TABLE 3. *Effect of unlabeled octanoic acid infusion on RAO oxidation*

Subj.	A. Control i.v. RAO			B. Postinfusion i.v. RAO				A − B % Diff.
	Dose, μc	DT, min	% Dose recovered	Octanoic acid load, g	Dose, μc	DT, min	% Dose recovered	
MP	2	2	19.3	1	2	3	18.8	+0.5
RA	2	2	19.4	1	2	2	16.2	+3.2
ET	2	2	19.6	1	2	3	20.3	−0.7
KB	2	2	22.8	1	2	3	24.2	−1.4
AH	2	2	22.4	1	2	3	21.5	+0.9
Avg.			20.7				20.2	

The average significance of comparison = $P > 0.7$.

acid [illegible]ion (column B) tests in the third group are compared on [illegible]. There was no statistically significant difference in the am[illegible]t of $C^{14}O_2$ recovered in these determinations.

DISCUSSION

The recovery of total radioactive carbon dioxide in the expired air following the administration of a tracer dose of labeled fatty acid has been previously used as an index to the rate of fat utilization in animals (5, 9, 10) and humans (2, 4). Using carbon 14-labeled palmitic acid, Brown was able to recover $C^{14}O_2$ within 2 min after intravenous injection in human volunteers (2). This rapid recovery of $C^{14}O_2$, which was also noted with the short chain fatty acid used in our experiments, is advantageous in the study of fatty acid absorption and oxidation.

In our studies $C^{14}O_2$ appeared in the expired air in 1–2 min after intravenous and 3–6 min after oral administration of the isotope. The difference between these two periods, i.e., the delay time after the oral dose minus that after the intravenous dose, represents the transit time of the isotope from the mouth to the portal venous system. Inasmuch as no significant difference in the recovery of $C^{14}O_2$ after oral and intravenous administration was noted, it would seem that the small amount of RAO used in these studies is completely absorbed from the intestinal tract. For this reason it would appear that the recovery of orally administered RAO may be used as an index of absorption in individuals without evidence of defective oxidation of fatty acids. Many studies in recent years have pointed out that the absorption transport and metabolism of fatty acids with 10 or less carbon atoms is handled differently than those with more than 10 carbon atoms (4, 6, 7). A number of experiments have determined *a*) that fatty acid absorption decreases as molecular weight of the fatty acid increases, *b*) that fatty acids with one or more double bonds are absorbed to a greater degree than are their saturated counterparts, and *c*) that the degree of absorption of any given fatty acid is greater if it is fed with less well-absorbed fatty acid (1, 3). These factors must be taken into consideration in determining whether octanoic acid absorption may be taken as indicative of normal fat absorption. Further studies are needed before this question can be answered fully.

Inhibition of long-chain fatty acid oxidation by glucose has been previously demonstrated by similar techniques (2, 7). Lossow and Chaikoff observed a marked inhibition of palmitic acid oxidation in rats after feeding a diet consisting of glucose, lactalbumin, corn oil, salt, and a vitamin mixture.

In the case of octanoic acid, however, only a minimal degree of inhibition was noted. Our studies performed after the administration of glucose, in a amount used in the standard glucose tolerance test, failed to reveal any significant inhibition of octanoate oxidation by this technique.

Intravenous infusion with moderate doses (1 g) of unlabeled octanoic acid likewise failed to inhibit the oxidation of C^{14}-labeled octanoic acid. It should be emphasized that with this 1-g dose of octanoic acid no significant change occurred in the shape of the curve or the location of the peak, as well as the total amount of $C^{14}O_2$ recovered in 50 min. This failure of inhibition of RAO oxidation may be due to the moderate dose of octanoic acid used or to the slow rate of infusion, i.e., approximately 17 mg/min. A larger dose, 2 g, of octanoic acid, however, was accompanied by flushing, nausea, joint and back pains in one volunteer, and resulted in a moderate depression of RAO oxidation. Because of these disturbing side effects no additional subjects were tested with this larger dose. The results suggest that oxidation occurred at the same rate after the 1-g octanoate infusion as with the tracer dose (199 μg) alone. We know from previous experiments that octanoate is rapidly metabolized (7), but the exact maximum rate of oxidation has not been clearly established in humans.

The studies reported here suggest that small doses of octanoic acid are virtually completely absorbed shortly after ingestion and that prompt oxidation is not substantially inhibited by moderate doses of glucose by mouth or by intravenous fatty acid. These features of octanoic acid metabolism could be exploited in the clinical study of fat absorption.

The authors are grateful to Binnie Rem for valuable technical assistance.

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P.A. Shore

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Platelet Damage Induced in Plasma by Certain Fatty Acids

The implication of lipids in the production of hypercoagulability of the blood has been recognized for a number of years. For example, the presence of certain lipid fractions has been shown to facilitate blood clotting *in vitro*, and hypercoagulability has been demonstrated in blood taken from human subjects after a meal of high fat content[1-3]. It has been shown recently that the addition of certain fatty acids to plasma accelerates the clotting time[4], and of especial interest are observations by Pooke and Connor that small quantities of long-chain saturated fatty acids, but not unsaturated compounds, evoke a marked hypercoagulability of blood in the thrombus-generator[5-7]. This instrument, originally described by Chandler, utilizes a revolving loop of plastic tubing into which blood is placed. The clot produced by this procedure has been shown to closely simulate thrombi occurring in human thromboembolic disease; that is, with a white head of platelets and leucocytes and a fibrin tail[8,9].

We have recently reported that rabbit plasma contains a factor which causes platelet damage as manifested by release of platelet histamine and serotonin, and that the plasma factor, though calcium dependent, acts even in the presence of a high concentration of heparin[10]. We have, therefore, examined the effects of fatty acids on platelets in plasma rendered incoagulable by heparin.

Platelet-rich plasma was prepared in siliconed glass centrifuge tubes as described previously[11]. Fatty acids were dissolved in distilled water with an excess of sodium hydroxide, and the *pH* was adjusted to about 7·8 with hydrochloric acid. Heparin was added to a final concentration of 80 μg/ml., followed by fatty acid solution. The tubes were then recalcified and incubated for 30 min at 37° C. Platelets were removed by centrifugation at 1,200 *g* for 30 min at room temperature. The histamine and, in some tubes, serotonin content of the supernatant plasma were then measured fluorometrically[12,13] and compared with the amine content of platelet-rich plasma.

Table 1. Effect of Fatty Acids on Release of Histamine from Rabbit Platelets under Various Conditions*

Condition	Histamine release (control = 1·0)†
Caprylic acid (50–250 μg/ml.)	1·0 (0·9– 1·3)
Myristic acid (25 μg/ml.)	2·2 (1·3– 2·9)
Palmitic acid (25–250 μg/ml.)	1·3 (1·0– 1·9)
Stearic acid (10 μg/ml.)	3·0 (1·9– 5·1)
Stearic acid (25 μg/ml.)	5·9 (3·0– 9·8)
Stearic acid (50 μg/ml.)	6·5 (3·6–10·4)
Arachidic acid (1–25 μg/ml.)	5·2 (2·4– 8·0)
Behenic acid (1–10 μg/ml.)	3·2 (2·4– 4·1)
Oleic acid (25–250 μg/ml.)	1·0 (0·8– 1·2)
Linoleic acid (100–250 μg/ml.)	1·1 (1·0– 1·3)
Linolenic acid (100–250 μg/ml.)	1·2 (0·9– 1·4)
Stearic acid (50 μg/ml.), no calcium	0·8 (0·6– 1·2)
Stearic acid (50 μg/ml.) cold (5° C)	0·7 (0·5– 1·2)
Stearic acid (25 μg/ml.) albumin solution	0·8 (0·6– 1·1)

* Fatty acids were incubated with rabbit platelet-rich plasma in the presence of heparin (80 μg/ml.), as described in text.

† Figures for histamine release denote ratio of percentage available histamine released in experimental tubes divided by histamine released in control tube (plasma + heparin + calcium) at 37°. Figures in parentheses denote range. Serotonin release (not shown) was about the same as histamine in all tubes measured.

As shown in Table 1, a marked release of platelet histamine occurred in tubes to which small quantities of stearic, arachidic, or behenic acids were added, while relatively little or no release occurred with lower molecular weight saturated fatty acids or with unsaturated fatty acids, even at much higher concentrations, indicating a considerable degree of specificity in the mechanism. Fig. 1 illustrates the effect of various concentrations of stearic acid. As shown in Table 1, no histamine release occurred in the absence of calcium ion or in tubes kept in the cold (5° C). The involvement of a plasma factor is suggested by the finding that stearic acid did not act on washed platelets suspended in buffered bovine serum albumin solution

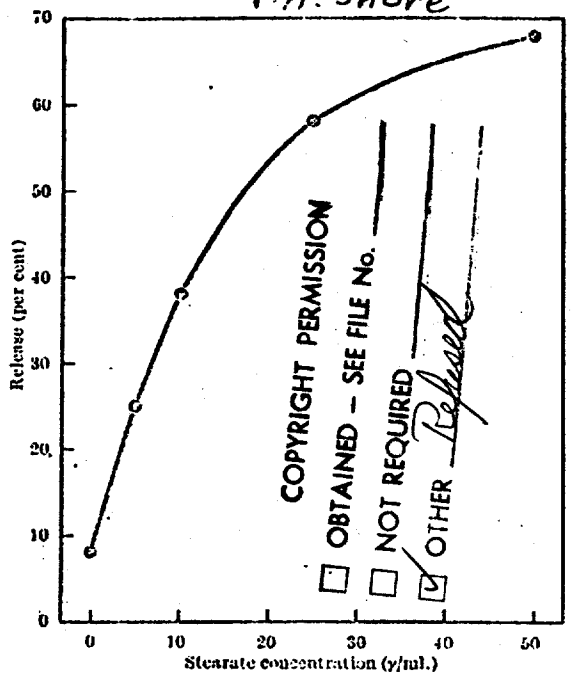

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Fig. 1. Effect of various concentrations of stearic acid on release of platelet histamine

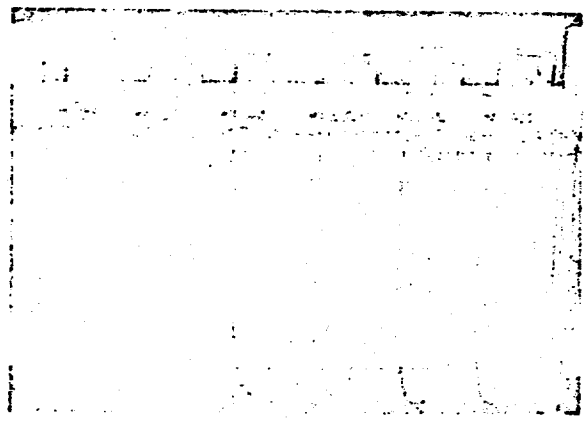

Fig. 2. Effect of stearic acid (25 μg/ml.) on rabbit platelets. Tubes were incubated and centrifuged as described in text. Left to right: (1) plasma—no stearate; (2) plasma + stearate—no calcium; (3) plasma + stearate; (4) plasma (50 per cent) + bovine serum albumin (50 per cent) + stearate; (5) plasma (25 per cent) + bovine serum albumin (75 per cent) + stearate; (6) bovine serum albumin + stearate. All tubes contained heparin (80 μg/ml.) and (except for No. 2) excess calcium ion. Figures for histamine release were: (1) 10, (2) 5, (3) 60, (4) 38, (5) 25, (6) 8 per cent

(Table 1). Fig. 2 illustrates the effect of stearic acid on platelets suspended in various combinations of plasma and bovine serum albumin solution. In the presence of plasma, stearate, and calcium ion, most of the platelets have adhered to the vessel wall, but platelet damage, as estimated visually or by histamine release, is minimal in the absence of any one of these factors.

The platelet damage described here occurs even in the presence of sufficient heparin to block the action of thrombin on fibrinogen or on platelets. The fatty acid effect thus may act through an early stage of the coagulation mechanism, one not affected by heparin. The mechanism possibly involves the action of Hageman factor, thought to be responsible for the effects of fatty acids on whole blood coagulation[6,7,14]. The heparin-insensitive mechanism of this type of platelet damage may be of importance in the aetiology of white thrombus formation and hypercoagulability. The experimental system described in this communication could provide a technique for the study of certain early coagulation steps.

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IMMUNOLOGY

Hæmolytic Complement and Serum Bactericidin

In a previous communication[1], serum bactericidin was related to a clotting factor extracted from fibrin. Several properties of this bactericidin are known to be shared by hæmolytic complement, such as inactivation by heating at 56° C for 30 min and removal by filtration through Seitz filter pads. Complement, like bactericidin, is absent from uncoagulated cell-free plasma[2]. Further investigations comparing the two serum activities were undertaken.

Both guinea pig and human sera served as sources of complement. Experiments were set up as follows. To fresh serum dilutions made up in 0·2 ml. of 0·9 per cent sodium chloride were added 0·2 ml. of 0·75 per cent sheep erythrocyte suspensions. Such cells had to be sensitized with immune hæmolysin when guinea pig serum was used, whereas with human serum this was not necessary. Tubes were incubated in a 37° C water bath for 1 h and read for complete hæmolysis. The smallest amount for this reaction or unit of complement was 0·005–0·01 ml. of guinea pig serum or 0·01–0·02 ml. of human serum. The difference between the action of guinea pig and human serum was explained by the finding that human serum normally contained both complement and sheep hæmolysin. Heating at 56° C for 30 min destroyed complement but left behind antibody which would bring about sheep cell lysis with guinea pig serum. The antibody could not be removed by repeated washings of the sensitized cells with saline. Its titre was determined by lysis with guinea pig serum (2 units of complement) as well as agglutination with anti-human globulin. Identical results were obtained. With most sera the minimal reactive dose was 0·005 ml. Normal human serum also contains sheep red blood cell agglutinins but of a much lower titre. A high titre of such agglutinins, as present in the sera of patients suffering from infectious mononucleosis, did not affect the hæmolysin titre.

No difference in the action of human and guinea pig complement was observed. Neither one was able to bring about the lysis of human Rh-positive cells sensitized with low-titred antibody, which strongly reacted with anti-human globulin. Like bactericidin, both were inactivated by lytic factor and heparin[1]. Only 0·01 ml. of potent lytic factor preparation was required to block the action of 2 units of complement. The reaction was immediate. It could be reversed by the addition of excess complement. Barium sulphate in 10 or 25 mg quantities absorbed in 10 min all complement from 0·1 ml. of human or guinea pig serum but did not affect serum bactericidin. Both complements also differed from bactericidin in thermostability at temperatures below 56° C, as reported previously[1].

Sera of a number of patients suffering from a variety of infections and organic diseases were analysed for complement and sheep hæmolysin. Considerable reduction of either one factor or both was noted in many instances; no consistent pattern emerged. High bactericidin levels often coincided with reduced complement activity. Guinea pig serum, though rich in complement, had no bactericidin.

From the results of this investigation as well as previous studies[1,4] it would seem that clotting factor extracted from fibrin, *C* reactive protein, serum bactericidin, and hæmolytic complement share a chemical grouping that arises immediately prior to or during fibrin formation. Indirect evidence[1] points to a steroid glucuronide. It would appear most likely that their differences in physico-chemical and physiological properties are due to association with different proteins.

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Specificity of Isoantisera against Leukæmic and Thymic Lymphocytes

THIS communication concerns the specificity of isoantisera prepared against leukæmic and against thymic lymphocytes of *AKR* mice, as determined by immune cytolysis[1-3]. Discovery of a strong antigen associated with the thymic lymphocytes of *AKR* mice is also reported. A preliminary report has been made[4].

For immune cytolysis of *L*4946 leukæmic lymphocytes of *AKR* mice, the small-scale assay system previously described in detail[2] was used. The only change was use of 100,000 cells per assay tube. Essentially the same cytolysis system was used for thymic lymphocytes, except that absorbed hamster serum at a final concentration of 10 per cent was used as source of complement[3].

Isoantisera were prepared by injection of leukæmic and of thymic lymphocytes of *AKR* mice into mice of strains *C57BL/6* and *C3HeB/Fe* (ref. 3). The cytolytic potencies of each of the four isoantisera were determined against *L*4946 leukæmic cells and against thymic lymphocytes (Table 1). Cytolytic potency is expressed as 100/cytolytic titre, where the cytolytic titre is the final concentration of isoantiserum (per cent) giving 50 per cent cytolysis of cells present in an assay tube. Specificity is expressed as the ratio of cytolytic potencies[3]; it refers to specificity against cell types, and has no direct bearing on the presence or absence of specific tumour antigens.

The relatively high potency of *C57BL/6* isoantisera for cytolysis of *L*4946 leukæmia (Table 1, *A*) correlates well with the complete resistance of *C57BL/6* mice to intraperitoneal transplantation of *L*4946 cells. The low potency of *C3HeB/Fe* isoantisera for cytolysis of *L*4946

Table 1. SPECIFICITY OF ISOANTISERA AGAINST LEUKÆMIC AND THYMIC LYMPHOCYTES

Isoantisera prepared against	Cytolytic potency for cytolysis of *L*4946 leukæmia (*AKR*)	Cytolytic potency for cytolysis of Thymic lymphocytes (*AKR*)	Specificity against *L*4946 leukæmia
A. Isoantisera prepared in *C57BL/6* mice.			
*L*4946 leukæmia	78	4	19·5
Thymic lymphocytes	24	450	0·053
Specificity against *L*4946 leukæmia	3·2	0·0089	366
B. Isoantisera prepared in *C3HeB/Fe* mice.			
*L*4946 leukæmia	4	310	0·01[illegible]
Thymic lymphocytes	3	450	0·00[illegible]
Specificity against *L*4946 leukæmia	1·33	0·76	1·8

INHIBITION OF RAT BLOOD SERUM N-ACETYL-L-TYROSINE ETHYLESTERASE BY FATTY ACIDS

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The nature of the inhibition of the cleavage of N-acetyl-L-tyrosine ethyl ester by a number of fatty acids (length of chain C_4, C_5, C_8, C_{10}, and C_{12}) was investigated. Evidence was obtained that these fatty acids act as competitive inhibitors of the reaction and that their inhibitory effect increases with the increasing length of their carbon chain. A procedure for spectrophotometric determination of N-acetyl-L-tyrosine, which is based on the difference in the solubility of the substrate and of the reaction product in ethyl acetate is described.

The mechanism of the cleavage of substrates containing nonpolar side chains by certain proteolytic enzymes has been interpreted in terms of the hypothesis postulating that these enzymes may bind the substrate by a hydrophobic group. The results of many kinetic studies on alpha-chymotrypsin[1], pepsin[2], carboxypeptidase, and leucine aminopeptidase[3] can be adduced in favour of this hypothesis. Its validity has been confirmed also by studies on the inhibitory effect of aliphatic alcohols[4,5] or fatty acids with long carbon atom chains[6,7]. In terms of this hypothesis it has been assumed that these compounds will compete by their nonpolar chain with the substrate in the formation of the complex with the enzyme and will thus act as competitive inhibitors of the cleavage. The obtained results have confirmed this assumption. Studies on the inhibition of pepsin[4] and leucine aminopeptidase[5] by lower alcohols have shown that the inhibitory effect depends on the length of the carbon atom chain.

Our preceding studies[8,9] were devoted to hydrolases cleaving the ester bond of a synthetic substrate, N-acetyl-tyrosine ethylester, which were inhibited by compounds whose structure is characterized by the presence of an electronegative group at one terminus of the molecule and of a lipophilic residue at the other[10]. Such a structure display among others fatty acids which show a merked inhibitory effect on the cleavage by N-acetyltyrosine ethylesterase. The present paper deals with studies on the kinetics of the inhibition reactions of a number of fatty acids and with the dependence of their inhibitory effect on the length of the aliphatic chain. A new spectrophotometric method for the determination of N-acetyl-L-tyrosine liberated by the enzymatic reaction is also described.

EXPERIMENTAL

Material and methods. N-Acetyl-L-tyrosin ethyl ester was prepared by Dr E. Kasafírek[11], Research Institute for Pharmacy and Biochemistry, Prague. N-Acetyl-L-tyrosine was prepared by alkaline hydrolysis of N-acetyl-L-tyrosine ethyl ester by 1N-NaOH. After treatment for several hours with alkali, the solution was neutralized with 1N-HCl and make up to the desired volume with water. Complete hydrolysis was checked by paper chromatography in the system butanol–acetic acid–water (3 : 1 : 1). Acetyl-tyrosine was detected by the diazo test according to Bray and coworkers[12]. The fraction of rat serum cleaving N-acetyl-L-tyrosine ethyl ester was prepared by electrophoresis in agar gel[9]. The absorbance measurement in the ultraviolet was performed in Universal Spektral Photometer, Zeiss Jena in a cuvet of *d* 0·502.

Spectrophotometric determination of N-acetyl-L-tyrosine. The fraction of rat serum (0·1 ml) was mixed with 0·2 ml of 0·5M Tris-HCl buffer at pH 8·5 and 0·2 ml of 0·025M solution of N-acetyl-L-tyrosine ethyl ester. The solution of the latter was prepared immediately before use by dissolving the compound first in acetone (1/10 of the desired final volume) and then adding the required volume of distilled water. The volume of the reaction mixture in the test tube was made up to 1 ml with water. The mixture was incubated in a water bath 20 minutes at 25°C. A blank solution not containing the enzyme was incubated under the same conditions. The enzymatic reaction was discontinued by the addition of 0·5 ml of 10% trichloroacetic acid and the formed precipitate was removed by centrifugation. The clear supernatant was neutralized by 1 ml of 2% Na_2CO_3 and the unhydrolyzed substrate was removed from the solution by three-fold extraction with 2 ml of ethyl acetate. The absorbance of the aqueous layer was measured at 274 nm. The measured extinction values were expressed in units of enzymatic activity using a calibration curve for N-acetyl-L-tyrosine (Fig. 1*b*). One unit is defined as the quantity of enzyme which will release 1 μmol of N-acetyl-L-tyrosine from the substrate in 20 minutes.

Kinetics of inhibition reactions. The inhibition reactions were studied as described above, with the exception of the purified fraction of rat serum which had been incubated first with the given inhibitor 10 min at 25°C. When the inhibition of N-acetyl-L-tyrosine ethylesterase by higher homologs of fatty acids (caprylic acid, capric acid, and lauric acid) was studied, the samples were filtered after deproteinization in order to remove the turbidity which had formed in the acid medium. The effect of the inhibitors was studied at four different substrate concentrations and at three different concentrations of the inhibitors. The results of the studies on the inhibition reactions were evaluated graphically using both the reciprocal plot of Lineweaver and Burk[13] and also a graph in which the dependence of the initial reaction rate was plotted against the ratio of initial rate to the concentration of substrate[14].

RESULTS AND DISCUSSION

N-Acetyl-L-tyrosine ethylesterase cleaves the ester bond at the carboxyl side of the substrate, thus liberating N-acetyl-L-tyrosine and ethanol. The absorption maximum of N-acetyl-L-tyrosine is identical with the absorption maximum of the uncleaved substrate and lies at 274 nm. The spectrophotometric determination of N-acetyl-L-tyrosine is based on the difference in the solubility of the reaction product and the uncleaved substrate in ethyl acetate and water.

As can be seen in Fig. 1*a*, the uncleaved substrate can be removed by three-fold extraction with ethyl acetate. At the same time, however, the content of N-acetyl-L-tyrosine in the solution decreases due to its partial solubility in ethyl acetate (Fig. 1*b*) during its extraction from the neutral

aqueous phase. The dependence of the extinction at 274 nm on the quantity of N-acetyl-L-tyrosine is linear (Fig. 1*b*) within the range of the molar concentrations used in the experiment. The described method cannot be used in studies on the inhibition by compounds which absorb in the region of the absorption maximum of N-acetyl-L-tyrosine, are not soluble in ethyl acetate, and cannot therefore be removed from the aqueous phase. The method is sufficiently sensitive and gives reproducible results. It could be therefore used for kinetic studies on the inhibition of N-acetyl-L-tyrosine ethylesterase by fatty acids.

We found that the inhibitory effect of fatty acids increases with the length of their chain (Fig. 2). When 0·1M solutions of fatty acids are used, butyric acid and valeric acids act as very little effective inhibitors while their homologs show increasing inhibitory activity. The maximal effect was observed with lauric acid. Inhibitory effect showed even palmitic acid, the quantitative evaluation, however, was difficult due to its high melting point and the results were therefore not included in the series.

The reaction of the cleavage of N-acetyl-L-tyrosine ethyl ester follows the kinetics of zero order only within a relatively narrow range of substrate concentration. The substrate concentration and the quantity of the inhibitors had to be chosen with

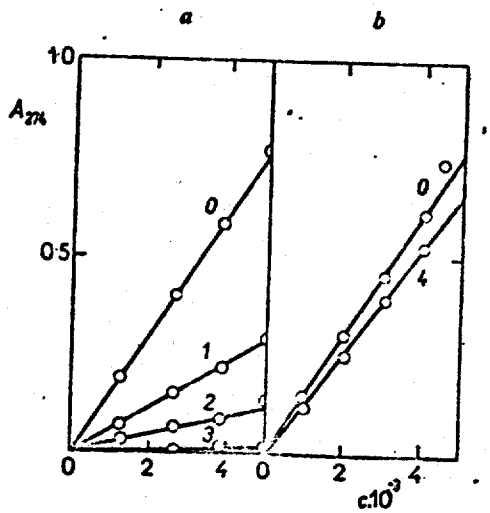

Fig. 1

Dependence of Absorbance at 274 nm on Molar Concentration of N-Acetyl-L-tyrosine Ethyl Ester (*a*) and N-Acetyl-L-tyrosine (*b*), respectively

0 Without extraction, *1* after first, *2* second, *3* third extraction with ethyl acetate, *4* after three-fold extraction with ethyl acetate; *c* molar concentration of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine, respectively.

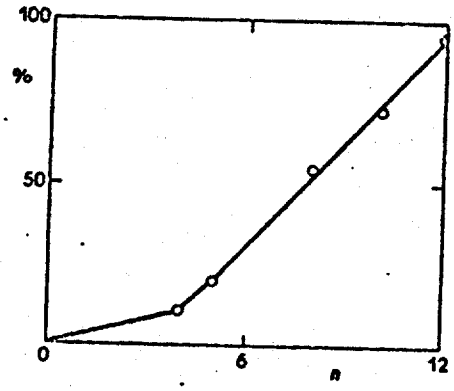

Fig. 2

Dependence of Inhibitory Effect on Length of Aliphatic Chain of Fatty Acids

n Number of carbon atoms of fatty acids; % of inhibition by 0·1M solutions of fatty acids.

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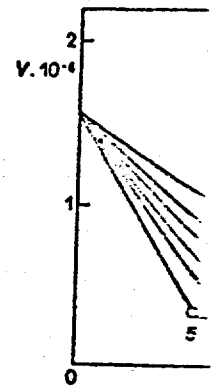

Fig. 3

Dependence of
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respect to this fact. The character of the inhibitory effect is obvious from Fig. 3 which shows the dependence of the initial reaction rate V on the ratio of initial rate V to substrate concentration S in a control experiment without inhibitor and in the presence of a series of fatty acids. The intersection of the straight lines with the ordinate gives the value of maximal reaction rate which is identical for all the reactions which were examined ($V_{max} = 1{\cdot}56 \,.\, 10^{-6}$M in 20 min). The inhibition constants of all inhibitors which were examined are given in Table I. Their value decreases with the increasing number of carbon atoms in the chain of the fatty acids. Fig. 4 shows the results of experiments with the inhibition of partly purified rat serum by different concentrations of valeric acid, expressed by the reciprocal plot of Lineweaver and Burk ($K_m = 3{\cdot}3 \,.\, 10^{-3}$M). As can be seen from both graphs, the fatty acids act as competitive inhibitors of the cleavage of N-acetyl-L-tyrosine ethyl ester.

The mechanism of the inhibition by fatty acids is most likely analogous to the action of aliphatic alcohols observed with pepsin[4] and leucine aminopeptidase[5]. Fatty acids, similarly to alcohols, compete with the substrate in occupying the hydrophobic binding site of the enzyme and react with the latter by the lipophilic moiety

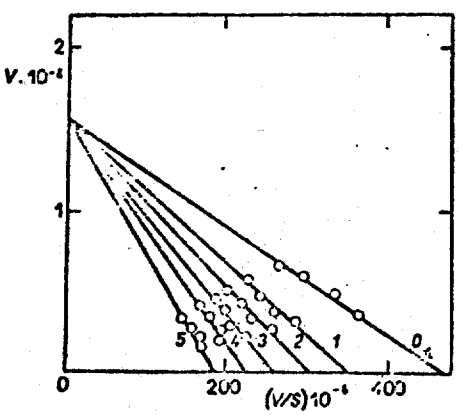

FIG. 3

Dependence of Initial Reaction Rate V on the Ratio of V to Substrate Concentration S

0 Without inhibitor, *1* in the presence of 0·2M butyric acid, *2* of 0·2M valeric acid, *3* of 0·005M caprylic acid, *4* of 0·005M capric acid, *5* of 0·0025M lauric acid. *S* concentration of substrate 2, 3, 4, 5 . 10^{-3}M; V is expressed in mol of N-acetyl-L-tyrosine liberated in 20 min.

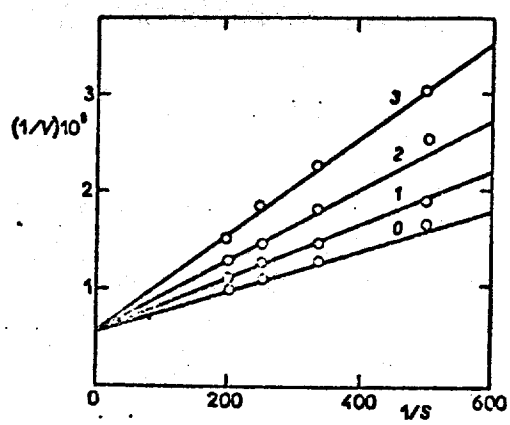

FIG. 4

Inhibition of N-Acetyl-L-tyrosine Ethylesterase of Partly Purified Rat Serum by Valeric Acid

0. Without inhibitor, *1* in the presence of 0·25M, *2* 0·20M, *3*. 0·15M valeric acid. *S* concentration of substrate: 2, 3, 4, 5 . 10^{-3} M; V is expressed in mol of N-acetyl-L-tyrosine liberated in 20 min.

TABLE 1

Inhibition Constants of Fatty Acids

Inhibitor	Molar concentration	K_i
Butyric acid	0·2	$1·4 . 10^{-1}$ M
Valeric acid	0·2	$9·8 . 10^{-2}$ M
Caprylic acid	0·005	$2·1 . 10^{-3}$ M
Capric acid	0·005	$1·4 . 10^{-3}$ M
Lauric acid	0·0025	$5·5 . 10^{-4}$ M

of their molecules. This conclusion is evidenced by data showing that the degree of competitive inhibition increases with the length of the aliphatic chain.

We may thus ask whether the inhibitory effect of fatty acids will manifest itself also in the process of the cleavage of the naturally occurring substrate of N-acetyl-L-tyrosine ethylesterase in the organism. The observation that in rats kept for a long period on a fat-rich-diet the content of N-acetyl-L-tyrosine ethylesterase of their serum[15] increases several times, seems to suggest that fatty acids might play a certain role also *in vivo*.

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Arch. Sci. Physiol. 21(2): 123-6 (1967)

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ÉTUDE «*IN VITRO*» DE L'OXYDATION DES ACIDES GRAS PAR DIVERS TISSUS DU JEUNE RAT SOUMIS A UN RÉGIME STEATOGÈNE (HYPOPROTÉIQUE, HYPERLIPIDIQUE) PRIVÉ DE CHOLINE ET DE VITAMINE B_{12}

II. LE TISSU HÉPATIQUE

par

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(*Reçu le 27 octobre 1966*)

INTRODUCTION

Dans un précédent travail (4) nous avons montré que le jeune rat soumis dès le sevrage à un régime hypoprotéique, hyperlipidique, privé de choline et de vitamine B_{12}, ne présente dans le rein aucun des signes de la carence en choline signalée par de nombreux auteurs. La signification de ce résultat a été discutée.

Dans le présent mémoire, nous étudions les effets de ce même régime au niveau du foie. Les recherches portent essentiellement sur les mesures de l'activité du système d'oxydation des A. G.

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RECHERCHES PERSONNELLES

1° Conditions expérimentales et techniques utilisées

Les expériences utilisent des rats mâles de 22 jours, pesant environ 40 grammes, de souche Wistar.

Les animaux sont divisés en 2 lots : le premier reçoit le régime complet (1) et le deuxième le régime stéatogène, privé de choline et de vitamine B_{12}. La composition de ces régimes a été donnée précédemment (2).

Après 28 jours de régime les animaux sont tués par décapitation, le foie est rapidement prélevé et homogénéisé. L'activité de l'octanoate-oxydase est mesurée dans les mêmes conditions que pour le rein (3) : par la consommation d'extra-oxygène et par le dosage de l'octanoate disparu. Nous avons, de plus, déterminé les corps cétoniques formés par la technique de WEISCHELBAUM et SOMOGYI (4).

RÉSULTATS EXPÉRIMENTAUX ET DISCUSSION

Les résultats exprimant la correspondance entre la consommation d'oxygène utilisé pour l'oxydation de l'octanoate et la quantité d'octanoate disparu sont résumés dans le tableau I.

TABLEAU I

Activité octanoxydasique de l'homogénat de foie de rat mâle Wistar âgé de 8 semaines

(1) Régime des rats	(2) Nombre d'expériences	(3) Consommation de O_2 en 20' (µM/mg N)			(4) Octanoate disparu	(5) Rapport $\frac{\text{extra } O_2 \text{ consommé}}{\text{Oct. disparu}}$
		T	E	T-E		
1	7	2,52 (± 0,19)	1,24 (± 0,13)	1,28 (± 0,11)	0,86 (± 0,10)	1,50 (± 0,10)
2	11	2,54 (± 0,15)	1,67 (± 0,07)	0,87 (± 0,07)	0,74 (± 0,05)	1,09 (± 0,04)

L'examen de ce tableau met en évidence les faits suivants :

1° L'oxygène consommé pour l'oxydation de l'oxtanoate (T — E) présente des valeurs plus faibles chez le jeune rat soumis au régime stéatogène que chez les témoins : 1,28 (régime 1) et 0,87 (régime 2), ceci représente une diminution de l'activité de l'octanoate-oxydase de l'ordre de 30 p. 100. Ce résultat montre que chez le jeune rat (*), comme chez l'adulte, la carence en choline s'accompagne d'une diminution du pouvoir oxydasique du foie à l'égard des A. C. Les diminutions observées chez l'adulte étaient toutefois plus importantes puisqu'elles étaient de l'ordre de 60 à 80 p. 100;

2° Alors que chez l'adulte la valeur du rapport $\frac{\text{extra-oxygène consommé}}{\text{octanoate disparu}}$ était de l'ordre de trois, correspondant à la transformation de l'octanoate en composés cétoniques, chez le jeune rat ce même rapport est égal à 1,50 pour les animaux témoins et 1,09 pour ceux soumis au régime stéatogène.

Ces valeurs ne correspondent pas à la transformation de l'octanoate disparu en composés cétoniques (rapport théorique 3) et encore moins à son oxydation totale (rapport théorique 11).

Pour discuter la signification de ce résultat nous avons mesuré la quantité de composés cétoniques formés correspondant aux quantités d'octanoate disparu. Les résultats sont réunis dans le tableau II.

TABLEAU II

Mesure de la formation de composés cétoniques correspondant à l'oxydation de l'octanoate par l'homogénat de foie de rat mâle Wistar âgé de 8 semaines

(1) Régime des rats	(2) Nombre d'expériences	(3) Octanoate disparu en 20' (µM mg N)	(4) C. cétoniques formés en 20' (µM mg N)	(5) Rapport $\frac{\text{C. Cétoniques}}{\text{Oct. disparu}}$
1	5	1,12 (± 0,07)	0,95 (± 0,09)	0,85 (± 0,08)
2	7	0,81 (± 0,08)	0,65 (± 0,08)	0,80 (± 0,06)

L'examen de ce tableau montre que la moitié environ de l'octanoate disparu s'est transformé en composés cétoniques.

(*) Nous avons vérifié que ces jeunes rats présentent également la stéatose hépatique.

Si donc seulement 50 p. 100 de l'octanoate disparu a été transformé en composés cétoniques, le problème se pose évidemment de connaître la destinée des 50 autres p. 100 disparus. Ont-ils subi une oxydation totale? S'il en était ainsi, le rapport $\frac{\text{extra-oxygène consommé}}{\text{octanoate disparu}}$ devrait être égal à 7, ce qui n'est point le cas puisque, comme il a été dit plus haut, ce rapport calculé à partir de l'extra-oxygène mesuré est de l'ordre de 1. Or, même en admettant que l'extra-oxygène mesuré ne corresponde pas à l'oxydation de l'octanoate disparu et en supposant que l'octanoate se substitue aux substrats de la respiration endogène, le rapport $= \frac{\text{oxygène consommé}}{\text{octanoate disparu}}$ calculé alors à partir de l'oxygène total consommé, est égal à 3. Ce chiffre est bien éloigné du 7 théorique. On ne peut retenir l'hypothèse selon laquelle les 50 p. 100 de l'octanoate disparu non transformés en corps cétoniques auraient subi une oxydation totale.

De plus, on constate que les valeurs théoriques d'oxygène nécessaires pour transformer 50 p. 100 de l'octanoate disparu en composés cétoniques correspondent aux valeurs expérimentales mesurées en prenant l'extra-oxygène comme étant la quantité d'oxygène utilisée pour effectuer cette transformation. En effet, chez les animaux soumis au régime stéatogène, la quantité expérimentale d'extra-oxygène est de = 0,970 µM en 20 minutes, alors que la quantité théorique est de 0,975 µM dans ce cas. De même, chez les animaux témoins, la quantité expérimentale d'extra-oxygène est de = 1,740 µm en 20 minutes alors que la quantité théorique d'oxygène nécessaire est ici de = 1,425 µM.

Cet ensemble de faits nous amène à conclure que la moitié environ de l'octanoate disparu s'est transformé en composés cétoniques, sans qu'il soit possible à l'heure actuelle de préjuger de la destinée de l'autre moitié disparue.

CONCLUSIONS

1° L'étude de l'oxydation de l'octanoate par l'homogénat de foie du jeune rat montre que seulement 50 p. 100 environ de l'octanoate disparu se transforme en composés cétoniques.

2° L'administration du régime hypoprotéique, hyperlipidique, privé de choline et de vitamine B_{12} a pour conséquence de diminuer l'intensité de l'oxydation de l'octanoate dans le tissu hépatique. Celle-ci présente toutefois les mêmes caractéristiques que chez l'animal témoin.

3° La signification de ces résultats est discutée.

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Free Fatty Acids and Monocarbonyls in Beer

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FREE FATTY ACIDS (FFA) and carbonyls are present in beer and apparently contribute to its aroma and flavor (13,14,18,25). Most of the series of free acids from methanoic through hexacosanoic acids have been reported in beer (18). Clarke *et al.* (5) reported a large number of free acids in Australian beer ranging from methanoic to hexacosanoic acids. Enebo (7) observed that beer from bottom yeast gave comparable levels of methanoic acid, but higher levels of ethanoic acid compared to beer from top yeast. Witt and co-workers (24,26,27,28) have used a colorimetric procedure to estimate total FFA in American beer and wort. In general, they found that the FFA concentration in beer was 3 to 4 times that in the corresponding wort. Äyräpää *et al.* (2) studied FFA in Swedish beers. They found that hexanoic and octanoic acids were quantitatively the most significant FFA. Sandegren and Enebo (21) observed that fatty acids of 8 to 12 carbon atoms predominate in beer. Suomalainen and Ronkainen (25) have briefly reviewed recent work on FFA and carbonyls in beer. Arkima (1) determined the relative amounts of the most important fatty acids in a number of different beers. He observed that isopentanoic, hexanoic, octanoic, and decanoic were the major FFA in beer. Arkima[1] investigated the lower fatty acids in bottom-fermented Finnish lager beer and observed that octanoic acid was the major FFA ranging from 5.6 to 8.1 mg./liter, and hexanoic acid varied from 3.1 to 5.8 mg./liter, whereas there were lesser quantities of other FFA.

In this paper, we report the quantification of FFA from butanoic through 9,12-octadecadienoic in beer and wort using a modification of the technique of Iyer *et al.* (16).

Methanal, ethanal, 2-propanone, propanal, butanal, isobutanal, isopentanal, pentanal, hept-4-one, deca-3-one, heptanal, and octanal are the principal aliphatic monocarbonyls reported in beer (18). Recently, Drews *et al.* (6), Ronkainen *et al.* (20), and Hashimoto (15) isolated the carbonyls in beer as their 2,4-dinitrophenylhydrazones. They subjected these to exchange reactions with keto acids or to oxidation with ozone, and the free aldehydes or volatile acids were studied with gas-liquid chromatography (GLC). Hashimoto (15) detected ethanal, propanal, 2-propanone, n-butanal, isobutanal, 3-methyl-1-butanal, 2-methyl-1-butanal, and three unknown carbonyl compounds. Ronkainen *et al.* (20) reported the presence of ethanal, isobutanal, isopentanal, pentanal, and butanal in order of decreasing concentration. Drews *et al.* (6) reported the presence of propanal, isobutanal, butanal, isopentanal, heptanal, and octanal in beer.

We report here some preliminary studies involving direct GLC of a crude extract of beer treated with 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazones and phenylhydrazones of aldehydes have been chromatographed by Soukup *et al.* (23) and by Fedeli and Cirimele (10,11). Recently, Fracchia *et al.* (12) quantified the carbonyls in automobile exhaust by GLC of the 2,4-dinitrophenylhydrazones. If direct GLC of the 2,4-dinitrophenylhydrazones from beer can be perfected, it would greatly simplify the quantification of beer carbonyls.

[1] V. Arkima, unpublished, cited in ref. 25.

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Experimental

Free Fatty Acids

Reagents. All solvents except Skellysolve B were analytical reagent grade, and most were distilled prior to use. Methanol and isopropanol (Baker and Adamson), n-butanol (Mallinckrodt), and Skellysolve B were distilled over potassium hydroxide. Anhydrous ethyl ether (Mallinckrodt) was passed through a basic alumina column. Reagent grade 85% phosphoric acid (Mallinckrodt) was untreated. Chromatographically pure standard fatty acids were obtained from Applied Science Laboratories, University Park, Pa., and from the Hormel Foundation, Austin, Minn. Butanoic, pentanoic, and heptanoic acids were purchased from Eastman Organic Chemicals and were found to be chromatographically pure. Silicic acid (Mallinckrodt-100 mesh) was treated as described by Iyer *et al.* (16).

Beer and wort samples. Beer samples from a pilot brewery were provided through the courtesy of B. A. Burkhardt of the Barley and Malt Laboratory, USDA, Madison, Wis. Beer prepared with corn adjunct generally served as the normal or control beer. In addition, an all-malt beer and a beer containing no hops were prepared. Beer was also analyzed before and after chillproofing. Three commercial beers were purchased at local markets and extracted by method I below. Wort was sampled prior to inoculation with yeast. The wort was filtered through a bacterial filter, adjusted to pH 7–7.5, and immediately freeze-dried. The dried wort was stored at −20°C. until analyzed. It was then reconstituted with distilled water to its original solids content and extracted by method II below.

Extraction, isolation, and esterification of FFA from beer and wort. The beer was filtered to remove carbon dioxide. Two alternative techniques of extraction of the beer were used.

Method I. The pH of each beer was adjusted to about 7.5 with dilute potassium hydroxide to convert FFA to their potassium salts. The beer was freeze-dried to yield a dry, fine powder. Ten grams of the beer powder were mixed well in a mortar with 10 ml. of saturated Na_2SO_4 solution to form a slurry. The mortar and pestle were chilled in an ice bucket and kept cool during the extractions. The pH of the slurry was reduced to 2.0 with 50% v./v. H_2SO_4 as determined on a separate batch of beer powder. About 2 mg. of each internal standard (pentanoic and pentadecanoic acids), accurately determined, was added to the slurry as an aliquot dissolved in a small amount of hexane. Medium sea sand (100–120 g.) was added to the slurry to form a wet granular mass. The FFA were extracted six times, each with 75 ml. of hexane containing 1% butanol. Each extraction required 10 min. of grinding with the pestle. Subsequently, a simpler extraction procedure was adopted.

Method II. To 350 ml. of degassed beer was added about 2 mg. of each internal standard (pentanoic and pentadecanoic acids), accurately determined and dissolved in 5 ml. of acetone, and 125 g. of NaCl was added to saturate the beer. The pH of the beer was adjusted to 2.0 with sulfuric acid (50% v./v.). The beer was then divided equally among four 250-ml. centrifuge bottles. Each portion was extracted six times, each with 50 ml. hexane containing 1% butanol. During each extraction, the portions were stirred vigorously for 10 min. with the aid of magnetic stirring bars. The emulsion formed between the beer and the solvent was separated by centrifuging at 2,000 × *g* for 10 min. In some beers, a gel was formed in the organic phase after the initial centrifugation. This gel could be readily disrupted with a glass stirring rod. The organic phase could then be recovered after centrifugation.

The extract from either alternative method was passed through a 5-g. silicic acid-KOH column described by McCarthy and Duthie (19) and as employed by Iyer *et al.* (16). A slight nitrogen pressure was applied to the column to obtain a flow rate of approximately 13 ml./min. The column was washed with 75 ml. ether to remove any lipid material, and the FFA were eluted with phosphoric acid in ether as described by Iyer *et al.* (16). An elution flow rate of 5 ml./min. was maintained. The solution containing the FFA was

diluted with an equal volume of methanol.

A yellow acid was extracted from the beer and became evident as a yellow band on the silicic acid-KOH column. The substance was eluted with the FFA and was carried through the remaining procedure. This material was tentatively identified from its absorption spectrum as an isohumulone by Etzer Chicoye of Miller Brewing Co. It did not appear to interfere with any peaks on the subsequent chromatogram.

Titration curves of butyric acid in the methanol-ethyl ether solvent were prepared with a Leeds-Northrup pH meter using miniature pH electrodes, No. 124138. These curves indicated an equivalence point at an apparent pH of 9.7. Therefore, the methanol-ether solution resulting from the beer was adjusted to an apparent pH of 11.7 with 1*M* methanolic KOH under nitrogen. This obviated an undesirable excess of KOH in the methanol-ether solution. An excess of base can possibly be removed by bubbling CO_2 through the solution. As the titration proceeded, insoluble phosphate salts precipitated. The phosphate salts were removed by centrifugation, and the clear supernatant containing the potassium salts of the FFA was evaporated to dryness as described by Iyer *et al.* (16). The acids were esterified by adding 1 ml. n-butanol and 0.05 ml. concentrated sulfuric acid to the salts and heating the mixture in a water bath at 100°C. for 2 hr. The ester solution was transferred to a Babcock skimmilk test bottle, shaken with 20 ml. of 1% sodium bicarbonate, and the esters were isolated over a concentrated sodium chloride brine (16). Jones and Davison (17) used a similar procedure to obtain quantitative recovery of butyl butanoate and higher esters. Extractions and analyses of beer without added internal standards were carried out exactly as described above. Reconstituted wort was also treated as described above. Solvent blanks were prepared as reported by Iyer *et al.* (16).

Completeness of extraction. The efficiency of the extraction procedure was studied with C^{14}-labeled butanoic, octanoic, and tetradecanoic acids. The radioactive reagents were obtained from Nuclear Research Chemicals, Inc., P. O. Box 6458, Orlando, Fla. The extractability of the fatty acids was determined for each isotope separately. Prior to the acidification step in the extraction, as described in method I, three microcuries of the sodium salt of 4:0, 8:0, or the potassium salt of 14:0 were added to the beer slurry. After 15 min., the slurry was acidified to pH 2.0, sand was added, and the fatty acids were extracted as before. Appropriate aliquots of the combined extracts were counted in a Packard Tri-carb 314 E liquid scintillation spectrometer. The activity of the added isotope was determined accurately by counting a suitable aliquot of standard fatty acid solution. In all cases, any quenching was corrected by adding C^{14} toluene as an internal standard of known activity. The standard error for the counting was less than 1%.

Test for ester hydrolysis during analyses. Iyer *et al.* (16) demonstrated that saponification of any lipid on the silicic acid-KOH column is negligible. To determine whether there was any acid hydrolysis of lipid at pH 2, we added 10 mg. of trilinolein to the beer powder and the FFA were extracted by method I. The FFA in the above beer were compared to the FFA in beer without added trilinolein. Also, Tween 80 (polyoxyethylene sorbitan monooleate) was used as a model for a water-soluble ester. About 60 mg. of Tween 80 was added to the beer powder. The quantity of FFA extracted from this beer was compared to the FFA in beer without added Tween 80, and with a blank sample of Tween 80 dissolved in toluene. In addition to the above tests, about 18 mg. each of ethyl butanoate and methyl hexanoate, dissolved in acetone, were added to 350 ml. of beer. The FFA were extracted by method II, isolated with the silicic acid-KOH column, and analyzed by GLC as before. The recovery of butanoic and hexanoic acids was compared to that for the corresponding beer without added ester. This would test for acid hydrolysis as well as saponification on the column.

Extractability of keto and hydroxy acids. Pyruvic, succinic, and lactic acids were dissolved in water in concentrations 1.5 to 2 times higher than re-

ported for beer by Enebo *et al.* (8). The solution was extracted according to method II. The extract was treated as described above, and the resulting n-butanol solution was analyzed by GLC.

GLC analyses. Butyl esters were analyzed by programmed temperature GLC using matched DEGS columns as detailed by Iyer *et al.* (16). The chromatograph was an Aerograph 1520, equipped with dual flame ionization detectors. The temperature was programmed linearly from 40° to 195°C. at a rate of 6°/min. Identification of esters was based on retention temperatures compared to standards and, in most cases, by adding known standards to the unknown samples and observing the increased increment of the unknown. Identification of major peaks was also confirmed in the same manner with columns packed with XE-60. In addition, identification of peaks was confirmed by plotting retention time against carbon number. Quantification was based on the area response of the unknowns as related to the area response of the internal standards. Pentanoic acid served as the internal standard for butanoic through decanoic acids, whereas pentadecanoic acid was the internal standard for the longer chain fatty acids. Standard butyl esters were analyzed after the standard FFA were passed through the silicic acid-KOH column and were esterified. The response factor for each acid relative to the appropriate internal standard was calculated as described by Bills *et al.* (4):

$$\text{Response Factor (Sought FA)} = \frac{(\text{wt. sought FA}) \times (\text{area internal std. ester})}{(\text{area sought FA ester}) \times (\text{wt. internal std.})}$$

When the above equation is rearranged, the concentration of sought fatty acid can be determined by using the appropriate response factor:

$$\text{Wt. (Sought FA)} = \frac{\text{response factor (sought FA)} \times (\text{area sought FA ester}) \times (\text{wt. internal std.})}{(\text{area internal std. ester})}$$

Peak areas were determined by multiplying the height by the width at half height. Overlapping peaks were estimated by approximating triangles essentially as described by Ettre (9).

Beer samples were analyzed before and after the additional of internal standards to determine if there were any peaks that interfered with 5:0 and 15:0.

Carbonyls

Reagents. Pyridine (Mallinckrodt), concentrated HCl (DuPont), and 2,4-dinitrophenylhydrazine (2,4-DNP) (Eastman) were used untreated. Carbonyl standards obtained from various supply houses were utilized without additional purification. Normal pentane (Phillips 66, commercial grade) and chloroform (Mallinckrodt) were passed through the reaction column of Schwartz and Parks (22). The 2,4-dinitrophenylhydrazones (2,4-DNPH) of carbonyl impurities in pentane were removed by passing the pentane through an alumina (neutral aluminum oxide, Brockman activity 1, Merck) column. The 2,4-DNPH impurities in chloroform were removed by double distillation. Both solvents were treated twice using these procedures. Ethanol was treated with 2,4-DNP by the method of Badings and Wassink (3). However, refluxing was carried out for 48 hr. or more followed by two distillations, the latter over sodium hydroxide. Water was double distilled over potassium permanganate.

Preparation of standards. Approximately 5 mg. of each saturated monoalkanal, varying in carbon chain length from 2 to 9, was dissolved in a liter of carbonyl-free ethanol-water (1:1 v./v.) which had been adjusted to pH 2.5 ± 0.1 with concentrated HCl. The solution was saturated with 2,4-DNP and was stirred for 2 hr. at room temperature. The 2,4-DNPH's were extracted with 3–350 ml. volumes of carbonyl-free pentane. Pentane was removed *in vacuo.* The residue was dissolved in a minimal amount of carbonyl-free chloroform. The solution was transferred to a screw-cap vial, and the chloroform was removed by a stream of nitrogen at room temperature. The 2,4-DNPH's were dissolved in diglyme for GLC. Sodium sulfate was added to the diglyme solution to remove water which seemed to interfere with the chromatography.

Treatment of beer. The contents of two 12-oz. bottles of beer were treated essentially as above. An equal volume of carbonyl-free ethanol was added to the beer, 2,4-DNP was added to saturation, and the reaction mixture stirred 2 hr. at room temperature. Blanks were run on the solvents, and a pentane extract was made of the untreated beer to determine if any extractables were chromatographing with the 2,4-DNPH's.

GLC analyses. An Aerograph 1520 equipped with a flame ionization detector was used. A 6 ft. by ⅛ in. stainless-steel column was packed with 5% SE-30 (Varian Aerograph) coated on 60/80 mesh, DMCS-treated, acid-washed Chromosorb W. Nitrogen and hydrogen flow rates were each 30 ml./min. Detector and injection port temperatures were each 300°C. Column temperature at injection was 100°C. and the temperature was programmed at 12°C./min. to 325°C. Unknown 2,4-DNPH peaks were identified primarily by retention temperature compared to standards. It was essential to condition the column prior to use by repeated injection of 2,4-DNPH standards. Apparently, adsorption sites on the column for the 2,4-DNPH's must be saturated.

Results and Discussion

Free Fatty Acids

Completeness of extraction. The average recovery of radioactive FFA for duplicate extractions was 90, 90, and 88% for 4:0, 8:0, and 14:0, respectively. These data indicate that the recovery of all acids during extraction was comparable. As a result, the internal standards were presumably extracted with the same efficiency as the other acids. Since the recovery is apparently the same for all FFA, the use of the actual amount of internal standard added to the beer in the above equations compensates for the 10% unrecovered FFA. Therefore, the data reported here represent the total FFA rather than the 90% recovered. The same beer was analyzed after extraction by method I and method II. The results were essentially the same for both extraction methods which indicated that extraction method II was yielding valid data.

Extractability of keto-, hydroxy-, and dicarboxylic acids. GLC indicated the absence of detectable hydroxy-, keto-, or dicarboxylic acids in the pentane-1% n-butanol extract of water containing pyruvic, lactic, and succinic acids. Apparently, there is no interference in the analyses from these acids.

Tests for ester hydrolysis during analyses. Beer is known to contain various esters. These esters are subjected to pH 2.0 during extraction and subsequently are exposed to the basic silicic acid-KOH column. Obviously, any hydrolysis of these esters during the analyses would increase the FFA content of the beer and would be undesirable. Three separate tests were made to establish the absence of ester hydrolysis. Firstly, extraction of beer powder containing trilinolein by method I indicated no increase in linoleic acid compared to beer powder without added linoleic acid. Secondly, extraction of beer powder containing Tween 80 by method I yielded an oleic acid content of 0.57 mg., whereas the yield of oleic acid from a blank containing an equivalent amount of Tween 80 was 0.61 mg. Thirdly, the butanoic and hexanoic acid content of beer extracted by method II was the same, within experimental error, in the presence and absence of ethyl butanoate and methyl hexanoate. It is estimated that with the latter method, 1% hydrolysis could be detected. In summary, these data indicate the absence of acid hydrolysis or of saponification of esters during the manipulations in the analyses.

GLC analyses. Table I shows the response factors used to quantify the FFA concentration in beer and wort. The response factors are close to unity as would be expected for a flame ionization detector. Table II shows the FFA concentrations in three commercial beers. There is a considerable variation in FFA concentration among the beers. For example, the total FFA varied from 19.5 mg./liter for beer I to 30.5 mg./liter for beer III. In most cases, the major FFA were butanoic, 3-methylbutanoic, hexanoic, octanoic, and decanoic acids. However, as would be

TABLE I
Response Factors Used to Quantify Free Fatty Acids in Beer and Wort

Acid	Response Factor[a]	Standard Deviation[b]
Butanoic	0.97	0.006
Pentanoic	1.00[c]	
Hexanoic	1.01	0.010
Heptanoic	1.07	0.006
Octanoic	1.09	0.020
Nonanoic	1.13[d]	
Decanoic	0.94	0.007
Undecanoic	0.95[e]	
Dodecanoic	0.97	0.013
Tridecanoic	0.97[e]	
Tetradecanoic	0.98	0.003
Pentadecanoic	1.00[c]	
Hexadecanoic	1.04	0.030
Octadecanoic	1.13	0.010
9-Octadecenoic	1.20	0.046

[a] Response factors for 3-methyl-butanoic and 9,12-octadecadienoic acids were those used for butanoic and 9-octadecenoic acids, respectively.

[b] Duplicate analyses for six determinations.

[c] Assigned.

[d] Obtained from a plot of log response factor *vs.* carbon number.

[e] Obtained from a plot of response factor *vs.* carbon number.

expected, beer I tends to be lower in the volatile FFA. These short chain fatty acids are, of course, very pungent and, as a result, probably contribute to the aroma of beer. We have added butanoic, hexanoic, and octanoic acids to distilled water, pH 4.3, at the approximate levels found in beer III. The solution had a distinct aroma characteristic of these FFA; however, the taste of the solution was quite bland. This is probably not a fair test of the contribution of these FFA to the aroma and taste of beer since these factors would probably be affected by carbonation, binding of FFA to beer solids, etc.

Table III shows the analyses of three pilot beers: a control beer containing corn adjunct, a similar beer containing no hops, and an all-malt beer. There is very little obvious difference among the beers. All beers contain similar levels of the short chain volatile fatty acids with butanoic, hexanoic, octanoic, and decanoic as the major FFA. These data suggest that the yeast is an important factor in the elaboration of the volatile FFA.

Analyses of beer prior to the addition of the internal standards during the preceding work indicated the absence of peaks interfering with the 5:0 and 15:0 internal standards. However, during subsequent work a peak became evident which interfered with 5:0. Analyses of solvents indicated an artifactual peak, presumably stemming from the n-butanol used in the esterification. Esterification with n-butanol-14% BF_3 (Applied Science), in an attempt to obviate this artifact, actually produced a larger peak that interfered with 5:0. Rather than change techniques, we elected to compensate for the artifactual 5:0 by first establishing an area for the artifact in relation to the 6:0 peak in beer containing no internal standards. The corresponding area was then determined in beer containing internal standard from the area of the 6:0 peak. This area was then subtracted from the 5:0 peak area to yield the "true" area of the internal

TABLE II
Free Fatty Acids in Commercial Beers[a]

Acid	Beer I	Beer II	Beer III
	mg./liter	*mg./liter*	*mg./liter*
Butanoic	0.91 ± 0.01	2.20 ± 0.01	2.37 ± 0.04
3-Methyl-butanoic	1.32 ± 0.06	1.17 ± 0.01	1.73 ± 0.02
Hexanoic	3.73 ± 0.03	4.34 ± 0.02	4.41 ± 0.09
Heptanoic	0.89 ± 0.04	0.17 ± 0.01	1.03 ± 0.01
Octanoic	6.77 ± 0.09	10.6 ± 0.2	11.7 ± 0.6
Nonanoic	0.43 ± 0.01	0.20 ± 0.01	0.41 ± 0.01
Decanoic	0.72 ± 0.01	1.34 ± 0.01	1.70 ± 0.06
Undecanoic	0.49 ± 0.02	0.34 ± 0.01	0.36 ± 0.01
Dodecanoic	0.33 ± 0.01	0.35 ± 0.01	0.25 ± 0.01
Tridecanoic	1.18[b]	0.72[b]	0.82 ± 0.04
Tetradecanoic	0.95 ± 0.03	2.5 ± 0.3	1.20 ± 0.01
Hexadecanoic	0.52 ± 0.01	0.19 ± 0.01	0.53 ± 0.01
Octadecanoic	0.39 ± 0.01	0.29 ± 0.01	0.51 ± 0.01
9-Octadecenoic	0.26 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
9,12-Octadecadienoic	0.63 ± 0.01	0.54 ± 0.02	.....
Total	19.5	25.6	30.5

[a] Average of duplicate analyses with maximum deviation.

[b] Single analysis.

TABLE III

Free Fatty Acids[a] in Control, No Hops, and All-Malt Beers

Acid	Control	No Hops	All-Malt
	mg./liter	*mg./liter*	*mg./liter*
Butanoic	1.13 ± 0.02	1.30[b] ± 0.01	1.27 ± 0.01
3-Methyl-butanoic	0.66 ± 0.01		0.52 ± 0.02
Hexanoic	4.02 ± 0.05	4.36 ± 0.04	4.63 ± 0.04
Heptanoic	0.84 ± 0.01	0.24 ± 0.01	0.65 ± 0.02
Octanoic	9.85 ± 0.05	10.1 ± 0.1	10.1 ± 0.3
Nonanoic	0.22 ± 0.01	0.20 ± 0.01	0.25 ± 0.01
Decanoic	1.71 ± 0.03	1.00 ± 0.01	1.36 ± 0.04
Undecanoic	1.01 ± 0.03	0.74 ± 0.01	0.81 ± 0.03
Dodecanoic	0.31 ± 0.01	0.32 ± 0.01	0.45 ± 0.02
Tridecanoic	0.75 ± 0.02	0.83 ± 0.03	1.40 ± 0.07
Tetradecanoic	0.65 ± 0.02	0.82 ± 0.02	0.76 ± 0.01
Hexadecanoic	0.54 ± 0.01	0.59 ± 0.03	0.60 ± 0.02
Octadecanoic	0.26 ± 0.01	0.26 ± 0.01	0.21 ± 0.01
9-Octadecenoic	0.23 ± 0.01	0.39 ± 0.01	.....
9,12-Octadecadienoic	0.44 ± 0.02	.....	0.26 ± 0.01
Total	22.62	21.15	23.52

[a] Average of duplicate analyses with maximum deviation.
[b] Butanoic + 3-methyl-butanoic.

standard peak which was then used to calculate FFA concentrations. Obviously, these additional calculations would affect the precision of the analyses and are undoubtedly a factor in the greater deviation observed in subsequent work.

We analyzed the FFA in beer and the corresponding wort to further establish the importance of yeast in elaboration of volatile FFA. Figures 1 and 2 show gas-liquid chromatograms of butyl esters of FFA in beer and wort, respectively. These chromatograms illustrate the greater abundance of short chain FFA in beer compared to wort. In both beer and wort, a number of peaks remain unidentified; only identified peaks were quantified. Since standards 9:0, 11:0, and 13:0 were not available, identification of these peaks was based on plots of retention time against carbon number. The plots resulting from the wort analyses indicated that the major peaks in the 11:0 to 14:0 region were not 11:0 or 13:0. Perhaps these peaks represent unsaturated or iso-fatty

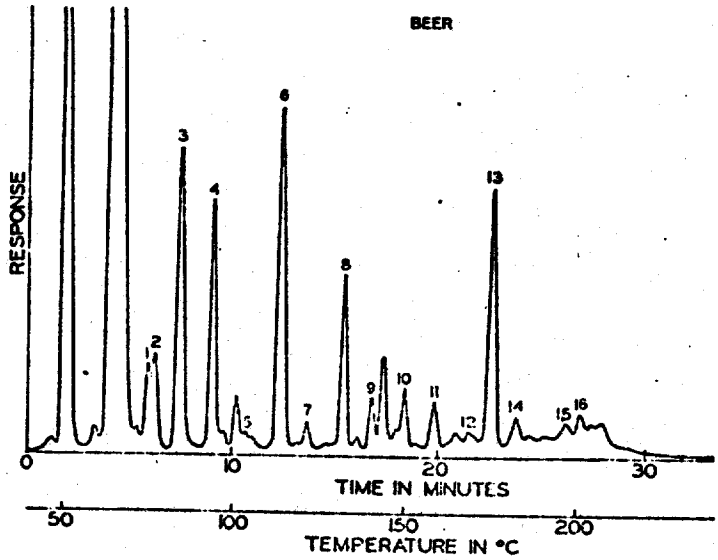

Fig. 1. Chromatogram of butyl esters prepared from free fatty acids in beer (DEGS column). 1, 4:0; 2, iso 5:0; 3, 5:0; 4, 6:0; 5, 7:0; 6, 8:0; 7, 9:0; 8, 10:0; 9, 11:0; 10, 12:0; 11, 13:0; 12, 14:0; 13, 15:0; 14, 16:0; 15, 18:0; 16, 18:1.

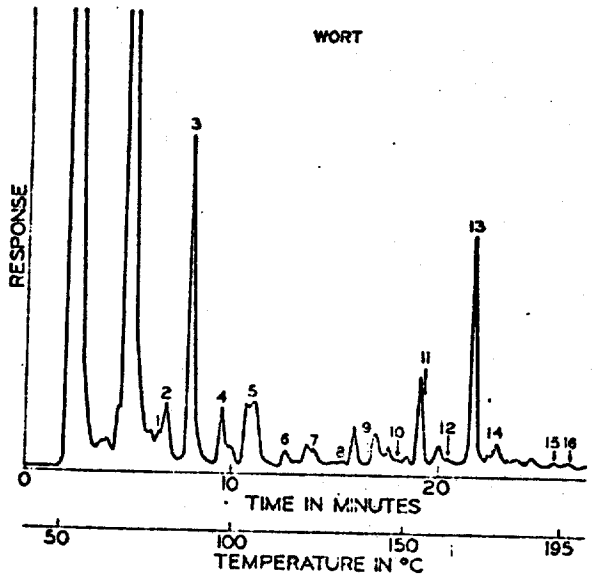

Fig. 2. Chromatogram of butyl esters prepared from free fatty acids in wort (DEGS column). 1, 4:0; 2, iso 5:0; 3, 5:0; 4, 6:0; 5, 7:0; 6, 8:0; 7, 9:0; 8, 10:0; 9, 11:0; 10, 12:0; 11, 13:0; 12, 14:0; 13, 15:0; 14, 16:0; 15, 18:0; 16, 18:1.

acids. Table IV shows the FFA in three pilot brews and the corresponding worts. Total FFA varied from 22.7 to 30.2 mg./liter in the beers, compared to 4.1 to 5.8 mg./liter in the worts. The wort tends to be lower in all FFA, particularly the short chain volatile FFA; however, in one wort sample, the 3-methyl-butanoic acid is higher than in the corresponding beer. These data emphasize the importance of the yeast in the generation of the volatile short chain FFA. Suomalainen and Ronkainen (25) have discussed the importance of the yeast in beer aroma development.

Analyses of beer before and after chillproofing indicated no essential difference in FFA contents.

TABLE IV
Free Fatty Acids in Beer and Wort

Acid	Concentration					
	Beer I[a]	Wort I[b]	Beer II[a]	Wort II[a]	Beer III[a]	Wort III[a]
	mg./liter	*mg./liter*	*mg./liter*	*mg./liter*	*mg./liter*	*mg./liter*
Butanoic	1.6 ± 0.2[c]	0.1[c]	2.2 ± 0.5[c]	0.2 ± 0.1[c]	1.19 ± 0.03[c]	0.41 ± 0.01[c]
3-Methyl-butanoic	1.2 ± 0.4[c]	2.2[c,d]	1.2 ± 0.1[c]	1.0 ± 0.1[c,d]	1.37 ± 0.03[c]	0.81 ± 0.01[c,d]
Hexanoic	4.7 ± 0.3	0.7	6.0 ± 0.5	0.6 ± 0.3	4.25 ± 0.01	0.9 ± 0.1
Heptanoic	0.6 ± 0.1[d]	0.5[d]	0.5 ± 0.2[d]	0.6 ± 0.3[d]	0.32 ± 0.03[d]	0.4 ± 0.1[d]
Octanoic	10.4 ± 0.3	0.2	13.0 ± 0.6	0.2 ± 0.1	11.1 ± 0.4	0.31 ± 0.05
Nonanoic	0.5 ± 0.2	0.3	0.5 ± 0.2	0.3 ± 0.1	trace	0.43 ± 0.08
Decanoic	2.6 ± 0.3	0.16	2.3 ± 0.2	0.09 ± 0.03	1.8 ± 0.1	0.23 ± 0.09
Undecanoic	0.8 ± 0.1	trace	1.3 ± 0.1	trace	0.85 ± 0.06	trace
Dodecanoic	0.9 ± 0.1	0.27	0.44 ± 0.03	0.13 ± 0.02	0.34 ± 0.09	0.18 ± 0.05
Tridecanoic	0.9 ± 0.1[d]	trace	1.1 ± 0.2[d]	trace	0.26 ± 0.04[d]	trace
Tetradecanoic	0.3 ± 0.1	0.23	0.11 ± 0.02	0.13 ± 0.03	0.03 ± 0.01	0.23 ± 0.09
Hexadecanoic	0.4 ± 0.1	0.6	0.4 ± 0.1	0.6 ± 0.3	0.31 ± 0.05	1.0 ± 0.3
Octadecanoic	0.3 ± 0.1	0.4	0.3 ± 0.1	0.17 ± 0.01	0.13 ± 0.01	0.5 ± 0.2
9-Octadecenoic	0.3 ± 0.2	0.09	0.3 ± 0.1	0.11 ± 0.03	0.16 ± 0.02	0.14 ± 0.04
Total	25.5	5.8	30.2	4.1	22.7	5.6

[a]Average concentrations and standard deviations for two to six different extractions are given each extraction chromatographed four to six times.
[b]Only one extraction was made.
[c]An approximate figure due to overlapping peaks on the chromatograms.
[d]The figure possibly includes more than one acid.

Carbonyls

Ethanol was of value in the treatment of beer by facilitating the solution of 2,4-DNP. The ethanol also prevented the formation of a gelatinous emulsion between the beer and pentane.

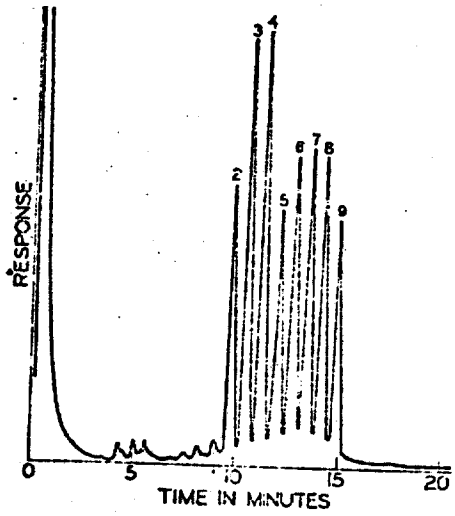

Fig. 3. Chromatogram of standard aldehyde 2,4-dinitrophenylhydrazones. Peaks 2 through 9 represent aldehyde carbon numbers 2 through 9, respectively (SE-30 column).

GLC analyses. Figure 3 shows a chromatogram of standard monoalkanal 2,4-DNPH's. The 2,4-DNPH's of the corresponding iso forms and methyl ketones have also been separated and, in general, exhibit slightly lower retention temperatures. However, the 2,4-DNPH of 2-propanone was not separable from propanal 2,4-DNPH. The 2,4-DNPH of unsaturated 2-enals, furfural, and diacetyl (mono form) have also been chromatographed.

It must be emphasized that the carbonyl analyses reported in this paper are of a very preliminary nature. Perfection of reliable means of studying the carbonyls in beer via direct GLC of the 2,4-DNPH's will require further work. GLC of the crude beer extract revealed peaks representing the saturated normal aldehyde 2,4-DNPH's of 2, 3, and 4 carbon atoms. Trace concentrations of isobutanal and isopentanal may also be evident. These are tentative identifications based only on retention temperatures compared to standards on one column packing.

A solvent blank in which water replaced the beer demonstrated impurities which were attributed to the 2,4-DNP reagent. Two of these impurities chromatographed at approximately the same retention temperatures as ethanal 2,4-DNPH and 2-propanone 2,4-DNPH.

A pentane extract of the beer-ethanol mixture without 2,4-DNP treatment yielded a chromatogram of a very broad low peak upon which were superimposed two small peaks. This broad peak exhibited a retention temperature similar to pentanal and represented considerable baseline drift. Addition of 2,4-DNP to this extract and subsequent GLC showed complete disappearance of the peak.

Extraction of the beer reaction mixture at pH 3 was important. Extracts obtained at higher pH values contained compounds which chromatographed at retention temperatures similar to hexanal 2,4-DNPH and octanal 2,4-DNPH.

In the above procedure, the 2,4-DNP reagent was added directly to the beer in the hope that a more complete recovery of carbonyl 2,4-DNPH's could be obtained compared to solvent extraction, steam, or vacuum distillation. As expected, the extract contained extraneous materials. Further progress in this area will probably require that the extract be cleaned up. Regardless of how the carbonyls are isolated, the direct GLC analysis of 2,4-DNPH's appears promising.

Summary

1. A GLC method for the quantification of FFA in beer and wort by the internal standard technique has been developed. Direct GLC of standard carbonyl 2,4-DNPH's has been accomplished on an SE-30 column. Preliminary studies were initiated on the 2,4-DNPH's of monocarbonyls in beer.

2. FFA analyses of three commercial beers indicated a variation in total FFA of 19.5 to 30.5 mg./liter. In most cases, the major FFA were butanoic, 3-methylbutanoic, hexanoic, octanoic, and decanoic acids. Hexanoic acid varied

from 3.7 to 4.4 mg./liter, whereas octanoic acid varied from 6.8 to 14.7 mg./liter.

3. FFA analyses of a control beer made with corn adjunct, a beer containing no hops, and an all-malt beer showed very small differences in individual FFA concentrations. These data tend to implicate the yeast as important in the elaboration of FFA.

4. FFA analyses of beer and the corresponding wort, in general, indicated that most of the butanoic, hexanoic, octanoic, and decanoic acids were evolved during the fermentation. These data suggest that the yeast is important in the elaboration of the volatile FFA.

5. Preliminary analyses of beer for monocarbonyl 2,4-DNPH's indicate, tentatively, the occurrence of ethanal, propanal, and butanal, and possibly, traces of isobutanal and isopentanal.

Acknowledgments

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SEPARATION AND DETERMINATION OF LOW-MOLECULAR-WEIGHT STRAIGHT-CHAIN (C_1–C_8) CARBOXYLIC ACIDS BY GAS CHROMATOGRAPHY OF THEIR ANILIDE DERIVATIVES

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(Received April 23rd, 1970)

SUMMARY

The analysis for formic, acetic, propionic, *n*-butyric, *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic acids in aqueous and non-aqueous mixtures by the gas chromatography of their anilide derivatives on sodium dodecylbenzenesulphonate coated on Chromosorb G is described. The derivatives of C_2–C_8 acids are formed in non-aqueous conditions via thionyl chloride treatment while the formic acid derivative is formed in aqueous conditions after pH adjustment. Optimum conditions for quantitative derivative formation are discussed. Variations of the pK_a values of the acids with derivative retention times and retention times and response factors with the number of carbon atoms are illustrated.

INTRODUCTION

Most of the methods described in the literature for the analysis of low-molecular-weight C_1–C_7 carboxylic acids by gas chromatography involve the direct injection of the acids into the column. This often necessitates the use of special combinations of stationary phases to obtain the optimum conditions for separation, but even then formic acid decomposition or lack of separation of it has been reported on some of these columns under the experimental conditions[1,2]. JANÁK AND KAPLANOVA[3] separated acetic and formic acids and water on a 1:3 mixture of bis(2-ethylhexyl) sebacate and citric acid coated on silanised Celite. MCKINNEY AND JORDAN[4] resolved formic, acetic, acrylic, propionic, *n*-butyric and isopentanoic acids and low-molecular-weight alcohols and aldehydes on 4% ethofat/2% isophthalic acid on Chromosorb T. A packing that will resolve a mixture of volatile fatty acids, C_1–C_5, has been reported by JACKSON[5], namely Ucon LB 550-X, sebacic acid and polypropylene glycol on 80/100 mesh AW Chromosorb W. Free formic and acetic acids in cigarette smoke have been determined by gas chromatography of their methyl esters on 20% Carbowax 20 M on 60/80 mesh Chromosorb P[6].

In the present work the anilide derivatives of formic, acetic, propionic, *n*-

butyric, *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic acids are chromatographed for quantitative determination on a single stationary phase coated on Chromosorb G. The derivatives are stable, non-volatile and easy to form. The method described lacks the disadvantages of the use of mixtures of stationary phases. The column packing remains stable and efficient over a long period of time because no free acids are passed through it.

EXPERIMENTAL

A Perkin Elmer Model F11 gas chromatograph equipped with dual flame ionisation detector was used for the analysis, and nitrogen served as the carrier gas.

Column preparation

2.5 g of sodium dodecylbenzenesulphonate (DBS) were dissolved in water–methanol (1:1) and the solution was added to 97.5 g of NAW 60/80 mesh Chromosorb G. After mixing by stirring the solvent was evaporated in the oven at 100° with occasional stirring until the solid appeared dry. It was cooled and packed by suction into 6 ft. (A) and 12 ft. (B) by 3 mm I.D. glass columns.

Column C. 1 g of potassium hydroxide was dissolved in 50 ml of methanol and added with more methanol to 99 g of NAW Chromosorb G. After mixing by stirring the solvent was removed. Then 97.5 g of the dry solid were added to 2.5 g of DBS dissolved in water–methanol, mixed thoroughly and the solvent removed as above in the oven. The solid was cooled and packed in a 6 ft. × 3 mm I.D. glass column (C).

The three packed columns A, B and C were conditioned at 220° for 18 h.

Intrument analytical conditions

The instrumental conditions and the columns employed for the various separations and determinations are listed in Table I below.

TABLE I

INSTRUMENTAL CONDITIONS FOR THE ANALYTICAL SEPARATIONS

Columns: (A) 6 ft. × 3 mm I.D. glass column packed with 2.5% (w/w) sodium dodecylbenzenesulphonate (DBS) on NAW 60/80 mesh Chromosorb G; (B) the 12 ft. × 3 mm I.D. version of column A; (C) 6 ft. × 3 mm I.D. glass column packed with 2.5% (w/w)-DBS on NAW 60/80 mesh 1% (w/w) base-loaded Chromosorb G.
The oxygen and hydrogen inlet pressures and the injection port temperature are the same for all the separations, *viz.* 26/18 lb./in.² and 250°, respectively.

Chromatography of the anilide derivatives of	*Instrumental conditions*
Formic and acetic or propionic or butyric acid using methyl stearate as internal standard	Columns A and C; oven temperature, 200°; nitrogen inlet pressure, 9 lb./in.² (Fig. 2)
C_1–C_8 (formic, acetic, propionic, *n*-butyric, *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic) acids, using methyl myristate as internal standard	Column B; oven temperature, 200°; nitrogen inlet pressure, 15 lb./in.² (Fig. 3)
	Columns A and C; oven temperature, 200°; nitrogen inlet pressure, 10 lb./in.² (Fig. 5)
Acetic, propionic, *n*-butyric and *n*-valeric acids (for quantitative separation)	Column B; oven temperature, 200°; nitrogen inlet pressure, 10 lb./in.² (Fig. 4)

GC DETERMINATION

Chemicals

The chemicals
The anilide derivati
SHAW[7] for retention

PROCEDURE

Preparation of the a chromatography

0.5 ml of the e
n-hexoic, *n*-heptoic,
50-ml stoppered tub
gently added. (The a
acid–thionyl chloride
The tube was placed
for 2 min in an ice
acetate was added a
solution of sodium b
the effervescence die
sec and left to stand
taken in a hypoderm

When the injec
ration the clear ethy
a 10–20 ml dry stop
derivatives could sta

RESULTS AND DISCUS

The anilide deri
quantitatively by th
chloride treatment. I
M) the anilides were
On the other hand fo
after thionyl chloride

$SOCl_2 + HCOC$

In aqueous medium, l
concentrations (0.05–

When these ac
scribed but must firs
layer. Though formic,
they were extracted
the amount extracte
distilled water and e
funnel, varied with t
butyric acids over a
39.3–41.4%, 45.0–50.
layer. The rest of the

Chemicals

The chemicals were used as obtained from BDH without further purification. The anilide derivatives of the carboxylic acids were prepared as described by OPENSHAW[7] for retention time determinations.

PROCEDURE

Preparation of the anilide derivatives of C_2–C_8 straight-chain carboxylic acids and chromatography

0.5 ml of the ethyl acetate solution of acetic, propionic, *n*-butyric, *n*-valeric, *n*-hexoic, *n*-heptoic, and *n*-octoic acids (0.5 *M* in each acid) were pipetted into a dry 50-ml stoppered tube containing 0.15 ml thionyl chloride. 0.5 ml aniline were then gently added. (The aniline was allowed to fall in drops directly onto the carboxylic acid–thionyl chloride mixture from a pipette without touching the walls of the tube.) The tube was placed unstoppered on a sand bath at 60–80° for 5 min and then cooled for 2 min in an ice bath. 10 ml of 0.1% (w/v) methyl myristate solution in ethyl acetate was added and the tube swirled round gently to mix. 10 ml of 1 *M* aqueous solution of sodium bicarbonate were added and the tube shaken unstoppered until the effervescence died down. It was then stoppered and shaken vigorously for 30 sec and left to stand for the layers to separate. 2 μl of the ethyl acetate layer were taken in a hypodermic syringe and injected into the instrument (columns B and C).

When the injection was not being done immediately after the derivative preparation the clear ethyl acetate layer was transferred with a clean dropping pipette to a 10–20 ml dry stoppered tube containing 2–5 g anhydrous sodium sulphate. The derivatives could stand in the solution for up to a week without deterioration.

RESULTS AND DISCUSSION

The anilide derivatives of a mixture of acetic to *n*-octoic acids may be prepared quantitatively by the method described only in non-aqueous conditions via thionyl chloride treatment. In aqueous medium and at the working concentrations (0.1–1.0 *M*) the anilides were hardly formed due to the ease of hydrolysis of the acid chlorides. On the other hand formanilide was hardly formed in non-aqueous medium especially after thionyl chloride treatment due to the reaction:

$$SOCl_2 + HCOOH \rightarrow CO + SO_2 + 2HCl$$

In aqueous medium, however, formanilide was obtained quantitatively in all working concentrations (0.05–1.0 *M*) even with thionyl chloride treatment.

When these acids are in an aqueous mixture they may be determined as described but must first be extracted into an organic (ethyl acetate or diethyl ether) layer. Though formic, acetic, propionic and *n*-butyric acids are miscible with water, they were extracted into the ethyl acetate layer in measurable amounts. However, the amount extracted individually into the organic layer, using equal volumes of distilled water and ethyl acetate and shaking vigorously for 1 min in a separatory funnel, varied with the amount of acid present, *e.g.* formic, acetic, propionic and *n*-butyric acids over a concentration range of 0.5 *M*–4.0 *M* in water were extracted 39.3–41.4%, 45.0–50.0%, 71.8–75.0% and 89.4–90.6%, respectively, into the organic layer. The rest of the acids gave 98–100% extraction when extracted individually.

Duplicate extractions of an equimolar (0.5 *M*) mixture of the C_2–C_8 acids gave a different extraction picture for each acid, *viz.* acetic acid, 15.5%; propionic acid, 30.3%; *n*-butyric acid, 36.8%; *n*-valeric acid, 39.2%; *n*-hexoic acid, 39.5%; *n*-heptoic acid, 39.5%; and *n*-octoic acid, 40.0%. The presence of mineral acids and salts also affected the carboxylic acid recoveries in the organic layer, so did the variation of the volume ratio of the organic and aqueous portions and the total acid concentration in the mixture. The investigation of these effects was not carried to the final conclusions as it might detract from the main object of this work. It must be pointed out that the recovery of each acid in a mixture would vary from situation to situation. It is therefore necessary to calibrate the extraction procedure for the acids in any given aqueous condition before proceeding with the derivative formation and gas chromatography.

The presence of water in the ethyl acetate solution of the acids reduces the amount of derivatives formed (hence lowers the sensitivity of the method) and distorts the chromatograms obtained from column B. Therefore after extraction of the acids from an aqueous medium the ethyl acetate layer must be dried with anhydrous sodium sulphate before being sampled for derivative formation.

Unknown acid mixtures may be determined by plotting calibration curves with standard mixtures, or by the use of the relative response factors (*i.e.*, weight of acid taken in mg/peak area *vs.* methyl myristate, Table III), using methyl myristate as the internal standard in both cases. The relative amount of each anilide formed (measured as the peak area ratio) in the mixture with any fixed amount of thionyl chloride present varies with the amount of aniline added (Fig. 1). Acetic, propionic and *n*-butyric acids on the one hand, and *n*-valeric, *n*-hexoic, *n*-heptoic and *n*-octoic acids on the other yield maximum amounts of derivatives at different concentrations of aniline. It was found reproducible and accurate enough in practice, however, to estimate first the number of mmoles of acid in an aliquot of the unknown mixture, and then to add thionyl chloride 1–2 mmoles in excess, followed by an amount of

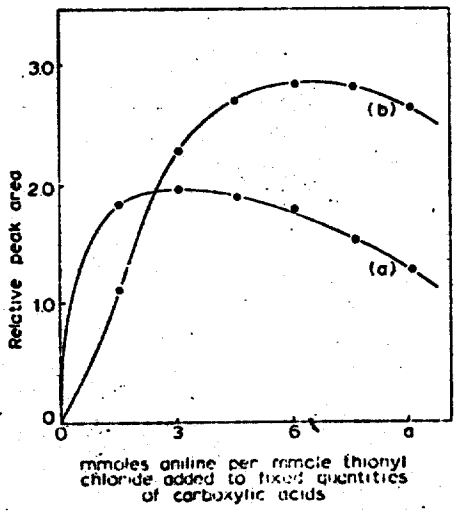

Fig. 1. Variation of quantity of derivative formed with increasing amounts of aniline. (a) *n*-Valeric–*n*-octoic acids; (b) acetic, propionic and *n*-butyric acids.

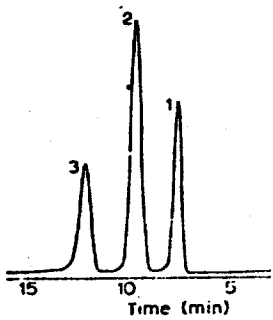

Fig. 2. Separation of a
standard on a 6-ft. 2.5

Fig. 3. Separation of a
2.5% DBS on Chromos
4 = *n*-valeric acid, 5 =
STD = methyl myrista

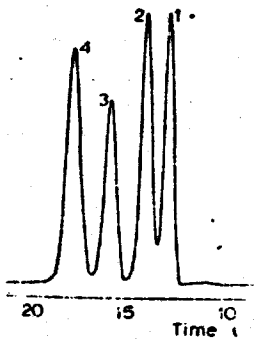

Fig. 4. Quantitative s
(3) acetic acid on a 1

Fig. 5. Separation of
DBS on Chromosorb
2 = acetic and *n*-buty
acid, 7 = *n*-octoic ac

C_8 acids gave a
propionic acid,
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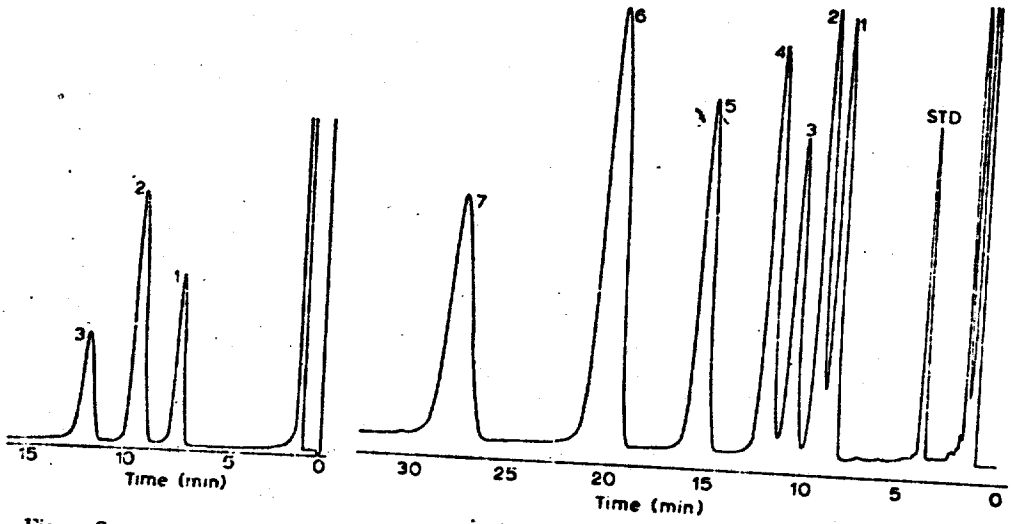


Fig. 2. Separation of acetanilide (1) and formanilide (3) with methyl stearate (2) as internal standard on a 6-ft. 2.5% DBS on Chromosorb G column.

Fig. 3. Separation of anilide derivatives of C_1-C_8 straight-chain carboxylic acids on a 12-ft. 2.5% DBS on Chromosorb G column. 1 = Propionic acid, 2 = *n*-butyric acid, 3 = acetic acid, 4 = *n*-valeric acid, 5 = *n*-hexoic acid, 6 = formic acid and *n*-heptoic acid, and 7 = *n*-octoic acid; STD = methyl myristate.

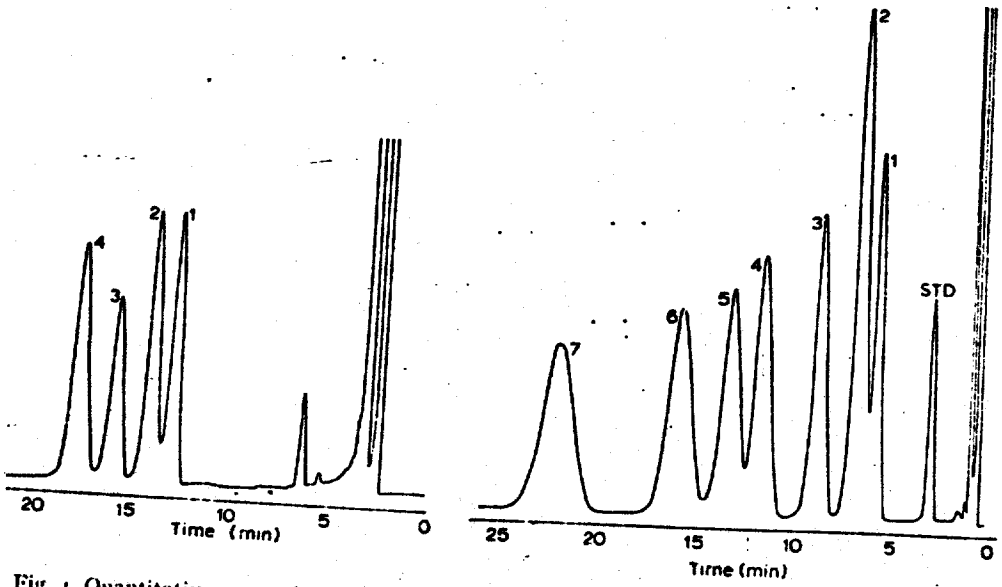


Fig. 4. Quantitative separation of anilide derivatives of (1) propionic acid, (2) *n*-butyric acid and (3) acetic acid on a 12-ft 2.5% DBS on Chromosorb G column. 4 = Valeric acid.

Fig. 5. Separation of anilide derivatives of C_1-C_8 straight-chain carboxylic acids on a 6-ft. 2.5% DBS on Chromosorb G column coated 1% w/w with potassium hydroxide. 1 = Propionic acid, 2 = acetic and *n*-butyric acid, 3 = *n*-valeric acid, 4 = *n*-hexoic acid, 5 = formic acid, 6 = *n*-heptoic acid, 7 = *n*-octoic acid; STD = methyl myristate.

TABLE II

RELATIVE RETENTION TIMES OF THE ANILIDES OF THE C_1–C_8 CARBOXYLIC ACIDS

Acid	pK_a (25°)[8]	*Relative retention time*	
		Column B	*Column C*
Formic (C_1)	3.77	6.34	5.0
Acetic (C_2)	4.76	3.21	2.41
Propionic (C_3)	4.88	2.57	2.0
n-Butyric (C_4)	4.82	2.86	2.41
n-Valeric (C_5)	4.81	3.79	3.16
n-Hexoic (C_6)	4.85	4.92	4.33
n-Heptoic (C_7)	4.89	6.64	6.08
n-Octoic (C_8)	4.85	9.42	8.66

aniline three to six times the number of mmoles of thionyl chloride added. The relative amount of aniline added to sample and standard aliquots must be the same.

The anilides of the C_1–C_8 carboxylic acids were separated on columns A, B and C. Heptoic and formic acid anilides were not separated on column B but they were

TABLE III

Acid	*Relative response factor*	
	Weight (in mg)	*Mmoles*
	Peak area	*Peak area*
Acetic	8.4	0.14
Propionic	8.14	0.11
n-Butyric	7.75	0.09
n-Valeric	7.14	0.07
n-Hexoic	6.96	0.06
n-Heptoic	7.02	0.05
n-Octoic	7.64	0.05

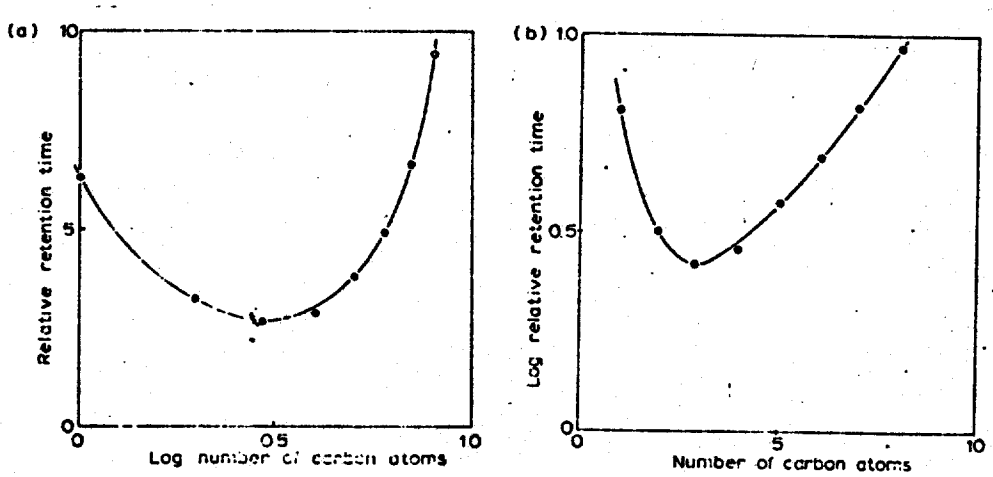
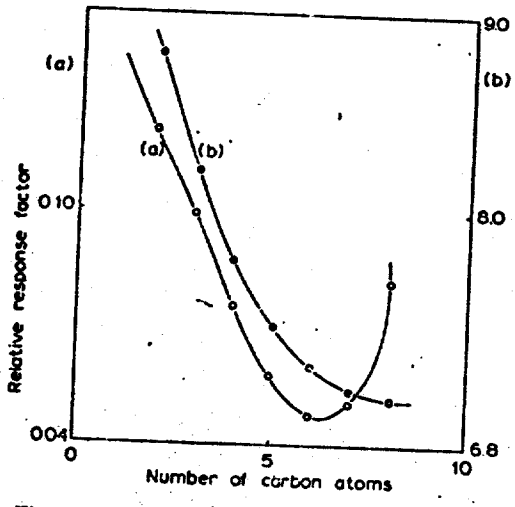


Fig. 6. (a and b) Variation of relative retention time with the number of carbon atoms on the carboxylic acids.

on columns A and C. Similarly, while *n*-butyric, acetic and propionic acid anilides were not resolved on columns A and C they were on column B. In the absence of *n*-butyric or acetic acid in the mixture the rest of the acids were quantitatively separated on columns A and C (Figs. 2–5).

The relative retention times (on columns B and C) and the response factors (on column B) of the acids are shown in Tables II and III, respectively. Their variations with the number of carbon atoms are illustrated in Figs. 6 and 7. The relative response factor decreases (in other words, the relative peak area per mmole of acid present increases) with increasing number of carbon atoms.

The effect of the pK_a of the acids on their relative retention times is not very clear. A plot of the pK_a (25°) *vs.* relative retention time produces the curves in Fig. 8. The pK_a values of formic, acetic, propionic and *n*-butyric acids lie on a straight line in relation to their retention times on column B.



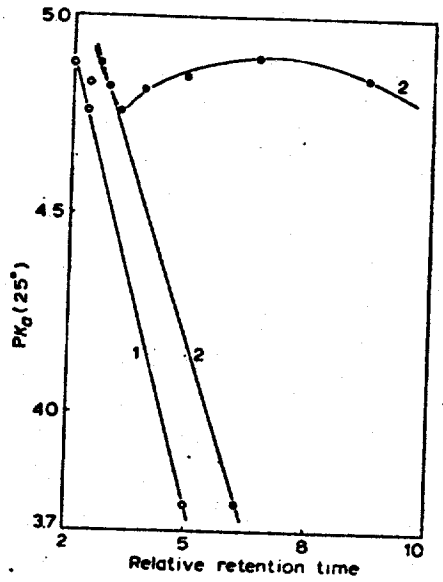


Fig. 7. Variation of relative response factors with the number of carbon atoms.
(a) (Weight of acid in mg/peak area) *vs.* number of carbon atoms
(b) (Mmoles acid taken/peak area) *vs.* number of carbon atoms

Fig. 8. pK_a (25°) *vs.* relative retention times of the anilides of the straight-chain C_1–C_6 carboxylic acids on columns B and C. (1) On column C; (2) on column B.

ACKNOWLEDGEMENT

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SUMMARY

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INTRODUCTION

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Bull. Soc. Chim. Biol. 9 : 483-500 (1927)

Recherches sur l'action inhibitrice des acides gras et, en particulier des acides non saturés, sur les phénomènes diastasiques,

155

par M. L. VELLUZ.

(7 mars 1927).

Dans un mémoire relatif au mécanisme d'action des diastases protéolytiques, MM. L. Hugounenq et J. Loiseleur [1] ont montré que, des deux éléments nécessaires aux phénomènes diastasiques étudiés, l'un — la micelle colloïdale — sert de vecteur à l'autre — cristalloïde ionisé — par sa polarité propre qui est susceptible de créer une électivité dans l'adsorption ionique. La suppression de l'un des facteurs entraine la disparition du phénomène diastasique : en l'absence d'électrolytes, la pepsine et la trypsine sont inactives ; en présence d'un colloïde inerte (gomme arabique) la protéolyse par les électrolytes (acide chlorhydrique, carbonate de sodium) n'est sensiblement pas modifiée [2]. Avec le glycogène, doué d'une polarité faible, on observe cependant une action catalytique incontestable.

Les recherches dont nous exposons les résultats dans ce mémoire montrent qu'on parvient à diminuer l'activité protéolytique de la pepsine en précipitant à la surface de sa micelle un acide gras, et en particulier un acide non saturé. Il est permis de supposer que cette action inhibitrice est due à la formation d'un film périmicellaire inerte qui diminue considérablement la polarité de la micelle ainsi enrobée. Nous avons obtenu des résultats analogues avec l'uréase du Jack Bean (*Canavalia ensiformis*).

∴

Si on dissout le sel sodique d'un acide gras dans une solution colloïdale quelconque (gomme arabique, glycogène, pepsine, trypsine, uréase) une fraction variable du savon est adsorbée à la surface des micelles. Cette adsorption s'exprime mathématiquement.

Cap Acid

par la loi de Gibbs, applicable à toute substance qui modifie la tension superficielle d'un liquide :

$$\Gamma = -\frac{c}{RT} \times \frac{\partial \sigma}{\partial c}$$

Γ = Accumulation du corps dissous par unité de surface de la couche superficielle ;
σ = Tension superficielle du solvant ;
c = Concentration du corps dissous dans le solvant ;
RT = Constante des gaz parfaits.

Dans le cas d'un savon, $\frac{\partial \sigma}{\partial c}$ est négatif : l'adsorption représentée ici par Γ est donc positive, ce qui revient à dire que le savon se concentre autour de chaque micelle colloïdale. Cette adsorption croît avec la température. Par addition de NaCl, il y a floculation du complexe colloïde-savon.

Nous avons recherché si cette adsorption d'un savon à la surface d'une micelle diastasique en modifie l'activité. Dans le cas de la pancréatine et de l'uréase, nous n'avons observé aucune modification.

Pour obtenir la formation d'un complexe colloïde-acide gras, il suffit d'acidifier dans une proportion convenable une solution colloïde-savon. L'acide gras libéré, s'il est insoluble ou très peu soluble dans l'eau, reste fixé sur la micelle colloïdale par un phénomène d'ordre vraisemblablement mécanique. La précipitation du colloïde par ses agents habituels de floculation entraîne l'acide gras fixé sur la micelle. La solution colloïde-acide gras présente une opalescence variable avec l'acide gras employé et surtout avec la concentration de cet acide. Pour éviter une floculation partielle ou totale du support colloïdal, il ne faut pas dépasser le plus souvent la concentration en acide gras de 20 p. 100 de colloïde sec.

Nous avons ainsi préparé des solutions colloïdales diastases-acides gras, stables et de concentrations variables en diastases et acides gras. Pour l'étude quantitative des enzymes ainsi traitées, nous avons dû opérer en milieu acide pour éviter la solubilisation de la pellicule. De plus, la nécessité d'une titration précise nous a conduit à opérer sur la pepsine et l'uréase.

Les acides gras utilisés ont été les suivants :

1° *Acides saturés* : Acides caproïque, œnanthylique, caprylique, pélargonique, caprique, palmitique, stéarique.

2° *Acides non saturés* : Acides crotonique, diméthylacrylique, oléique, ricinoléique. Acides non saturés de l'huile de lin, préparés selon la méthode indiquée par G. Bertrand [3] et que nous désignerons par convention : acide linolénique (indice d'iode 188).

I. — Etude de la pepsine.

Nos essais ont été faits sur la pepsine commerciale dite « en paillettes » de titre 1.000. Les digestions ont été faites à 37° sur de l'ovalbumine fraîche, coagulée par la chaleur et divisée en cubes sensiblement identiques. Nous n'avons pas employé l'ovalbumine colloïdale pour éviter les phénomènes d'adsorption secondaire susceptibles de modifier l'interprétation de nos résultats. L'azote aminé soluble, libéré par la protéolyse dans un temps variant de 6 à 16 heures a été déterminé selon la technique de Sörensen au formol.

A. — *Forte concentration en acides gras par rapport à la concentration en pepsine :*

Pepsine	0,5 p. 1000
Acide chlorhydrique	0,5 —
Ovalbumine coagulée	5 p. 100
Acides gras	3 p. 1000

—	Azote soluble libéré après 16 h. d'étuve (1)
Témoin	4,3
— + acide caproïque	4,2
— — œnanthylique	3,1
— — caprylique	3,4
— — pélargonique	2,3
— — caprique	2,4
— — palmitique	1,1
— — stéarique	1
— — oléique	1
— — linolénique	1
— — ricinoléique	1

Tous les acides gras diminuent donc le pouvoir protéolytique de la pepsine. L'action inhibitrice croît avec la condensation en carbone, de C^6 à C^{18}.

Il est naturel d'attribuer ce fait à la solubilité correspondante

(1) En mgr. p. 100 cm³ de liquide essayé.

des acides dans l'eau, solubilité qui décroît aussi quand la teneur en carbone s'élève. Si la micelle colloïdale est enrobée par un acide gras légèrement soluble dans l'eau, le film périmicellaire se prête plus facilement à des échanges par diffusion ou osmose, échanges qui conditionnent l'action diastasique.

A même condensation en carbone — C^{18} —, aucune différence n'est observée entre acides saturés ou non saturés.

Toutefois, cet essai est, à lui seul, peu probant par suite de l'instabilité du colloïde pepsine-acides gras aux concentrations considérées. Après 16 heures d'étude une floculation partielle se produit et le simple entraînement de la pepsine pourrait expliquer à lui seul la lenteur de la protéolyse. Néanmoins les variations relatives dans cette floculation sont déjà susceptibles d'être interprétées.

B. — *Faible concentration en acides gras par rapport à la concentration en pepsine* :

Nous avons employé dans ce cas deux échantillons de pepsine de titres 100 et 1000 et nous avons remarqué que plus le titre d'une pepsine est faible, plus la concentration en acides gras peut être forte sans qu'il y ait floculation.

Théoriquement, en effet, si l'adsorption de l'acide par la micelle pepsique, est un phénomène d'ordre électrostatique, il ne peut y avoir formation du colloïde pepsine-acides gras dont les constituants sont tous deux électronégatifs. Pour une pepsine de titre faible, la présence de colloïdes étrangers, sans polarité propre, permet la fixation des acides gras, d'où l'apparence d'une concentration limite bien supérieure. Pour une pepsine de titre très élevé, la formation du complexe d'adsorption est vraisemblablement due à un phénomène purement mécanique, dont l'intensité n'est jamais comparable à celle d'un phénomène d'ordre électrique.

Pepsine	2,5 p. 1000
Acide chlorhydrique	2,5 —
Ovalbumine coagulée	5 p. 100
Acides gras	0,4 p. 1000

	Azote soluble libéré après	
	6 h. d'étuve peps. titre 1000	16 h. d'étuve peps. titre 100
	—	—
Témoin	6,5	7,2
— + acide caproïque	6,4	6,6
— — œnanthylique	6,4	6,6

Témoin + acide caprylique	6,2	6,3
— — pélargonique	5,9	6,3
— — caprique	5,9	6,1
— — palmitique	5,6	5,8
— — stéarique	5,6	5,8
— — oléique	5,1	2,8
— — ricinoléique	3,6	1,2
— — linolénique	3,7	1,3

Nous retrouvons ici la relation entre le pouvoir d'inhibition d'un acide gras et sa teneur en carbone ; mais, contrairement à l'essai précédent, pour une même teneur en carbone — C^{18} — il y a une différence entre acides saturés et non saturés. Nous croyons pouvoir interpréter ce fait en faisant intervenir les théories de Langmuir [4] relatives à l'adsorption et à l'étalement des acides gras.

Selon cet auteur :

1° La couche d'adsorption d'un corps à la surface de séparation de deux phases est unimoléculaire ;

2° Pour un acide gras insoluble dans l'eau, la surface recouverte par cette couche unimoléculaire (force d'étalement) est fonction de la présence dans sa molécule de groupements chimiques déterminés.

Langmuir a ainsi déterminé la surface moléculaire de plusieurs acides gras en C^{16} et C^{18}, et ses résultats sont les suivants :

Surface couverte par une molécule d'acide	palmitique	21×10^{-16} cm²
—	stéarique	22×10^{-16} —
—	oléique	46×10^{-16} —
—	linolénique	65×10^{-16} —
—	ricinoléique	110×10^{-16} —

En présence d'acide chlorhydrique à 0,1 p. 100, les surfaces sont légèrement augmentées :

Acide palmitique	37×10^{-16}
— stéarique	35×10^{-16}
— oléique	67×10^{-16}
— ricinoléique	126×10^{-16}

Les acides non saturés couvrent tous un espace plus grand, par molécule, que les acides saturés. La molécule de l'acide ricinoléique apparaît comme la plus étendue, par suite de la présence de trois groupes « actifs » : COOH, OH alcoolique, liaison éthylénique.

Considérons alors une micelle colloïdale partiellement enrobée par un acide gras : pour une même concentration de cet acide l'enrobement sera d'autant plus parfait que l'on passera de l'acide stéarique à l'acide ricinoléique. Il en sera de même pour l'ensemble des surfaces micellaires. Le pouvoir inhibiteur de l'acide variera dans le même sens que sa capacité d'étalement.

Nos résultats vérifient tout à fait cette hypothèse dans le cas de la pepsine. Toutefois, les théories de LANGMUIR ne sont rigoureusement applicables que dans des conditions physico-chimiques bien déterminées qui sont loin d'être les nôtres. La complexité des facteurs dans les essais biologiques ne permet de rechercher qu'un ordre de grandeur dans l'étude des phénomènes. C'est seulement cet ordre de grandeur que nous cherchons à retrouver dans le parallélisme entre le pouvoir inhibiteur et la force d'étalement.

Si la concentration de l'acide gras est telle qu'avec l'acide de force d'étalement minima toutes les micelles sont enrobées, on ne doit plus observer de différences entre acides saturés et non saturés, exception faite des variations possibles dues à des états physiques dissemblables. C'est le cas du premier essai mentionné plus haut.

C. — *Influence de la concentration en acide gras :*

Essai I. : Pepsine..... 0,5 p. 1000
Acide chlorhydrique..... 0,5 —
Ovalbumine coagulée..... 5 p. 100

	Azote libéré après 16 heures d'étuve
Témoin.....	6,1
— + acide oléique 0,2 p. 1000.....	5,3
— + — 0,5 —	5
— + — 3 —	0,8 (floculation partielle).

Essai II. : Pepsine..... 3 p. 1000
Acide chlorhydrique ..... —
Ovalbumine coagulée ..... 5 p. 100

	Azote libéré après 6 heures d'étuve
Témoin.....	10,9
— + acide palmitique... 2 p. 1000.....	10,3
— + acide ricinoléique.. 2 —	6,5
— + — .. 10 —	0,4 (floculation).

Le pouvoir inhibiteur croît avec la concentration en acide ; pour la concentration de 2 p.1.000 on observe toujours une différence notable entre l'acide palmitique et l'acide ricinoléique.

Les essais suivants montrent que la concentration en acide chlorhydrique ne modifie pas l'allure du phénomène.

Essai : Pepsine..... 3 p. 1000 (titre 100).
Ovalbumine coagulée..... 5 p. 100
Acide ricinoléique ..... 1 p. 1000

	Azote libéré après 20 heures d'étuve	
	acide chlorhydrique 3 p. 1000	acide chlorhydrique 6 p. 1000
Témoin	9,1	9,3
— + acide ricinoléique	3,1	2,9

Nous avons observé de plus qu'en l'absence d'électrolytes la fixation d'acides gras sur la micelle pepsique ne lui confère aucun pouvoir protéolytique. De même, la présence d'un colloïde, gomme-arabique-acide gras, ne modifie pas la protéolyse par les électrolytes seuls, ce qui parait montrer que l'enrobement par une pellicule d'acides gras ne confère à la micelle aucune polarité propre.

Si l'acide gras est fixé sur une micelle autre que la micelle diastasique (gomme arabique), sa présence ne modifie pas la digestion pepsique :

	Azote libéré après 20 heures d'étuve
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) 3 p. 100 + acide chlorhydrique 3 p. 100	8,2
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) + gomme arabique 2 p. 100.....	8,1
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) + gomme arabique 2 p. 100 + acide oléique 3 p. 1000.....	7,9
Ovalbumine coagulée 5 p.100 + pepsine (titre 100) + gomme arabique 2 p. 1000 + acide ricinoléique 3 p. 1000.....	7,9
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) + gomme arabique 2 p. 100 + acide linoléique 3 p. 1000.....	7,9

Enfin si l'acide considéré est soluble dans l'eau, il n'influence en rien l'activité protéolytique :

	Azote libéré après 6 h. d'étuve
Ovalbumine coagulée 5 p. 100 + pepsine (titre 1000) + acide chlorhydrique 1 p. 1000	6,2
Ovalbumine coagulée 5 p. 100 + pepsine (titre 1000) + acide crotonique 1 p. 1000	6,1
Ovalbumine coagulée 5 p. 100 + pepsine (titre 1000) + acide diméthylacrylique 1 p. 1000	6,2

D.— *Fixation de l'acide gras sur l'albumine en digestion.*

La technique suivie pour l'obtention du colloïde ovalbumine-acide-gras est un peu différente de celle donnée plus haut pour la pepsine. Nous avons préparé une solution alcaline d'ovalbumine.

Ovalbumine fraiche	50 cm³
Soude normale, q. s. p.	250 —

Le savon a été dissous dans cette solution alcaline et nous avons ajouté de l'acide chlorhydrique en quantité suffisante pour saturer la soude et décomposer le savon.

Essai I. : Pepsine	1 p. 1000 (titre 100)
Acide chlorhydrique	—
Ovalbumine colloïdale	3 p. 100
Acides gras	2 p. 1000

	Azote libéré après 20 h. d'étuve
Témoin	13,2
— + acide caproïque	11,9
— — oenanthylique	11,8
— — capryllique	11,8
— — caprique	11,2
— — palmitique	11,2
— — stéarique	11,2
— — oléique	11
— — linolénique	10,3
— — ricinoléique	10,3

Essai II (sans pepsine) + Acide chlorhydrique	3 p. 100
Ovalbumine colloïdale	1 p. 100
Acides gras	2 p. 1000

	Azote libéré après 20 h. d'étuve
Témoin	2 mgr.,1
— + acides gras	1 mgr.,6

Ces résultats sembleraient donc montrer que la protection de l'albumine par les acides gras est d'autant plus accentuée que les acides sont plus riches en carbone ou non saturés. Mais, en raison des faibles différences observées, nous ne croyons pas devoir en faire état.

II. — Etude de l'uréase.

Le dynamisme de la diastase du Jack Bean (*Canavalia ensiformis*), étudié par Van Slyke [5], paraît être différent de celui de la pepsine. En effet, selon cet auteur, l'hydratation de l'urée par l'uréase comporte deux phases successives :

1° Combinaisons de l'enzyme avec l'urée :

$$[E] + [U] \longrightarrow [EU] \qquad (1)$$

réaction de vitesse constante c ;

2° Rupture de cette combinaison, l'urée étant libérée sous forme de carbonate d'ammoniaque

$$[EU] \longrightarrow [E] + CO^2 + NH^3 \qquad (2)$$

réaction de vitesse constante d.

L'application de la loi de Guldberg et Waage aux équations (1) et (2) a permis à Van Slyke de formuler la loi mathématique suivante :

$$t = \frac{1}{E}\left[\frac{1}{c}\,\mathrm{Log.}\,\frac{a}{a-x} + \frac{x}{d}\right]$$

où t est le temps de décomposition d'une quantité x d'urée, E la concentration de l'enzyme et a la quantité d'urée présente par unité de volume au début de la réaction.

L'expérience vérifie parfaitement cette formule si on prend toutefois la précaution d'opérer en présence de solutions tampons qui maintiennent le pH constant. Les vitesses de réactions c et d varient en effet beaucoup avec le pH ; elles passent par un maximum pour pH = 7 et tendent vers zéro quand l'alcalinité du milieu augmente par suite de l'accumulation du carbonate d'ammoniaque formé.

Nous avons préparé une uréase d'une activité suffisante par simple macération de 10 gr. de farine de Jack Bean dans 100 gr. d'eau distillée à 15°-20° pendant 2¼ heures, sous toluène. La

solution obtenue après centrifugation constitue une solution mère dont nous avons utilisé une partie aliquote dans nos essais.

L'acide gras a été fixé sur la micelle d'enzyme selon la même technique que pour la pepsine. Le colloïde uréase-acide gras, précipité par addition de 5 fois son volume d'acétone et le floculat renferme intégralement l'acide fixé.

Les solutions utilisées ont été uniformément amenées au pH = 4,7, par dilution convenable d'acide acétique normal et acétate de soude normal, à parties égales. Dans tous nos essais le pH n'a pas varié de plus de 0,15, du début à la fin de la fin de la réaction. Chaque solution mère n'ayant été utilisée que pour une série d'essais, nos résultats ne doivent pas être considérés en valeur absolue, mais seulement en valeur relative par rapport aux témoins correspondants.

Le dosage de l'ammoniaque formé a été effectué, sur 100 cm³ de liquide essayé, selon la méthode au formol.

I. — *Même concentration en uréase et acides gras :*

Essai : Uréase..... 0,5 p. 1000
Urée..... 5 p. 100
Acides gras..... 0,5 p. 1000

	Ammoniaque formée en 30' (1)
Témoin.....	52
— + acide linolénique.....	48
— — ricinoléique.....	42
— — oléique.....	46
— — stéarique.....	36
— — palmitique.....	36

Il y a inhibition de l'enzyme comme dans le cas de la pepsine, mais ici l'allure du phénomène est inversée : l'inhibition est plus forte pour les acides saturés — solides — que pour les acides non saturés liquides. Nous attribuons ce fait au dynamisme particulier de l'uréase : première phase où l'enzyme se combine avec le substrat. Cette combinaison est vraisemblablement plus difficile en présence d'une pellicule solide, moins sensible à l'osmose qu'une pellicule liquide.

II. — *Faible concentration en acides gras par rapport à la concentration en uréase :*

(1) En mgr. p. 100.

Essai : Uréase..... 2,5 p. 1000
Urée..... 5 p. 100
Acides gras..... 0,2 p. 1000

	Ammoniaque formée en 30'
Témoin.....	11½
— + acide palmitique.....	11½
— — stéarique.....	11½
— — oléique.....	98
— — ricinoléique.....	88

Le parallélisme entre le pouvoir inhibiteur et la force d'étalement réapparaît comme dans le cas de la pepsine.

III. — *Rôle de la concentration en acide gras :*

Essai : Uréase..... 1 p. 1000
Urée..... 1 p. 100

	Ammoniaque formée en 30' — Concentration p. 1000 en acide gras					
	0,2	0,1	0,08	0,04	0,02	
Acide ricinoléique.....	33	38	38	36	42	
— oléique.....	35	39	40	42	42	témoin
— linolénique.....	32	34	35	39	42	42 mgr
— palmitique.....	40	39	40	42	42	
— stéarique.....	40	39	40	42	42	

La concentration minima où le pouvoir inhibiteur apparaît, est en relation avec la force d'étalement.

Les résultats donnés par les essais précédents sont donc sensiblement symétriques de ceux obtenus avec la pepsine. Il importe toutefois de remarquer que dans le cas de l'uréase le phénomène est plus complexe que dans le cas de la pepsine. Ici, trois facteurs interviennent :

1° Etat physique, solide ou liquide, de l'acide employé ;
2° Force d'étalement de l'acide ;
3° Pression osmotique de la solution d'urée.

Ce dernier facteur nous a paru particulièrement important. Si on imagine, en effet, une micelle enrobée d'une pellicule insoluble, mais cependant sensible à la pression osmotique, les échanges à travers cette pellicule sont fonction :

1° de son épaisseur ;

2° de la pression osmotique du liquide extérieur.

Or, pour une même concentration en acide (1), l'épaisseur d'une pellicule est d'autant plus faible que la force d'étalement est plus forte. Ainsi, les échanges par osmose seront plus faciles pour une pellicule d'acide ricinoléique que pour une pellicule d'acide oléique. Les 2 facteurs, force d'étalement et épaisseur de la pellicule, ont deux effets distincts : film périmicellaire mince, mais enrobant un nombre important de micelles, ou film périmicellaire de plus forte section, mais n'enrobant qu'un petit nombre de micelles. Il est dès lors permis de supposer que, selon la pression osmotique du liquide extérieur — c'est-à-dire la concentration en urée —, le pouvoir inhibiteur variera soit dans le même sens que la force d'étalement, soit en sens contraire.

Nous avons fait dans ce but une série d'essais où la concentration de l'urée varie de 0,1 à 12 p. 100.

Essai : Uréase..... 1 p. 1000
Acides gras..... 0,4 p. 1000
Temps..... 30 minutes.

Concentration en urée p. 100	Ammoniaque formée (*)			
	Témoin	Acide palmitique	Acide oléique	Acide ricinoléique
12	29.1	29	27,3	25.6
10	29.1	29	27,3	26,6
5	29,1	27,1	24,2	21,3
1	23	21,2	19,4	20.3
0,5	19	18,4	15,6	16.8
0,2	12	11,1	9,7	10,5
0,1	7,1	6,8	6,3	6,6

(*) Exprimé ici en cm³ de soude décinormale.

(1) Concentration faible par rapport à la concentration en diastase.

D'après ce tableau, on voit que :

1° Avec une concentration en urée au-delà de 1 p. 100, la pression osmotique est assez forte pour que l'épaisseur du film périmicellaire n'intervienne plus. L'action inhibitrice croît alors avec la surface totale enrobée, c'est-à-dire avec la force d'étalement.

2° Avec une concentration en urée égale ou inférieure à 1 p. 100, l'épaisseur du film intervient suffisamment pour que l'acide oléique devienne plus inhibiteur que l'acide ricinoléique.

Nous avons tenté d'appliquer à l'essai précédent l'équation de Van Slyke

$$t = \frac{1}{E}\left[\frac{1}{C}\ \mathrm{Log.}\ \frac{a}{a-x} + \frac{x}{d}\right]$$

qui peut encore s'écrire :

$$0{,}4343\ c = \frac{d}{Etd-x}\ \mathrm{Log.}\ \frac{a}{a-x}$$

La constante d est déduite des valeurs de x et t déterminées quand la concentration de l'urée est assez forte pour que la vitesse de réaction en soit indépendante : $\frac{1}{c}\log.\frac{a}{a-x}$ devient négligeable

$$t = \frac{1}{E} \times \frac{x}{d} \qquad d = \frac{x}{Et}$$

on remplace, dans l'équation de Van Slyke, les lettres par leurs valeurs numériques et on recherche la constance de C.

Les valeurs de c obtenues dans les 4 tableaux suivants montrent que si l'équation de Van Slyke est applicable pour l'essai témoin, elle ne l'est plus quand l'uréase est enrobée par un acide gras. La cinétique de la réaction est modifiée, surtout aux concentrations fortes en urée. C'est dans le cas d'un film périmicellaire de faible épaisseur (ac. ricinoléique) que la vitesse c (vitesse de combinaison de l'enzyme avec l'urée) reste sensiblement constante.

***

En résumé, alors que la fixation d'un savon sur une micelle diastasique telle que la pancréatine ou l'uréase reste sans effet appréciable sur son activité, la fixation d'un acide gras sur la pepsine et l'uréase la modifie notablement. Dans certaines limites de concentration, les acides non saturés sont plus actifs que les

1. Essai : témoin.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	29,1	9,7	
10	3330	29,1	9,7	
5	1665	29,1	9,7	
1	333	23		0,493
0,5	166,5	19		0,499
0,2	66,6	12		0,487
0,1	33,3	7,1		0,460
	exprimés en cm3 soude décinormale			

2) Essai : témoin + acide palmitique.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	29	9,66	
10	3330	29	9,66	
5	1665	27,1		0,513
1	333	21,2		0,37
0,5	166,5	13,4		0,455
0,2	66,6	11,1		0,426
0,1	33,3	6,8		8,43
	exprimés en cm3 soude décinormale			

3) Essai : témoin + acide oléique.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	27,3	9,1	
10	3330	27,3	9,1	
5	1665	21,2		0,117
1	333	19,4		0,287
0,5	166,5	15,6		0,319
0,2	66,6	9,7		0,313
0,1	33,3	6,3		0,414
	exprimés en cm3 soude décinormale			

4) Essai : témoin + acide ricinoléique.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	25,6	8,53	
10	3330	25,6	8,53	
5	1665	21,3		0,24
1	333	20,3		0,40
0,5	166,5	16,8		0,43
0,2	66,6	10,5		0,42
0,1	33,4	6,6		0,43
	exprimés en cm3 soude décinormale			

acides saturés et ce fait parait en relation avec leur force d'étalement.

L'intérêt de ces résultats nous parait dépasser celui d'une expérimentation réalisée *in vitro*. En effet, par l'antagonisme du pH des liquides interstitiels (pH 7,3), et cellulaire, (pH 6), une fixation analogue à celle que nous considérons ici est possible *in vivo*.

Il est permis d'imaginer l'existence de complexes diastases-savons dans les liquides de pH > 7 et celle de complexes diastases-acides gras dans les liquides de pH < 7. Dans les conditions normales du métabolisme des acides gras, il est probable que l'association enzyme-acide contribue à l'équilibre diastasique. Ne peut-on pas alors supposer que la déviation de ce métabolisme (combustion ou production excessives, prédominance des acides non saturés sur les acides saturés) modifiant l'activité des complexes diastases-acides gras alors qu'elle affecte peu celles des complexes diastases-savons, il y aura dysharmonie dans le jeu diastasique complet ?

MM. Hugounenq et Loiseleur [6] ont montré que le chimisme d'une micelle colloïdale amphotère peut présenter des actions diastasiques inverses pour des variations du pH de part et d'autre du point isoélectrique. Dans le cas des associations enzymes-savons enzymes-acides gras, l'activité diastasique pourra être modifiée sans variations du pH. Il est d'ailleurs possible que l'existence de ces couples diastasiques constitue pour l'organisme un mécanisme régulateur en quelque sorte « tampon » du système diastasique. On peut schématiser ce fait de la façon suivante :

1° pH constant (< 7) :

Solution tampon : acide oléique + oléate de soude

$$\text{acide oléique} \underset{+\ \text{acide}}{\overset{+\ \text{alcali}}{\rightleftharpoons}} \text{oléate de soude} \quad (1)$$

En présence d'une micelle diastasique :

$$\begin{array}{ccc} \textbf{inhibition} \longleftarrow & \text{enzyme} + \text{oléate Na} & \\ & \downarrow + \text{acide} \qquad \uparrow + \text{alcali} & \\ & \text{enzyme} + \text{ac. oléique} \longrightarrow & \textbf{activation} \end{array}$$

(1) Les flèches indiquent un déplacement de l'équilibre comme on le comprend en général dans les solutions tampons : les concentrations, des constituants sont toujours telles que la constance du pH est assurée.

2° pH variable ($\lessgtr 7$) :

$$\text{enzyme} + \text{savon} \underset{\text{pH} > 7\ \text{(activation)}}{\overset{\text{(inhibition)}\ \text{pH} < 7}{\rightleftharpoons}} \text{enzyme} + \text{acide gras} \quad (1)$$

Nous suggérons l'intervention possible d'un mécanisme analogue dans l'équilibre ou le déséquilibre du biochimisme diastasique cellulaire et il est permis de croire à l'intérêt de recherches poursuivies dans cette voie, notamment en ce qui concerne la physiopathologie du cancer.

Quoiqu'il en soit, il apparait ici que les liaisons éthyléniques ont une importance considérable, quoique indirecte, dans le jeu des diastases associées *in vivo* à des acides gras ou à leurs sels. Nous avons déjà souligné le rôle qu'elles jouent vraisemblablement dans la narcose [7]. Alors que les lipides saturés constituent un potentiel énergétique d'ordre essentiellement *statique*, les acides non saturés paraissent jouer un rôle *dynamique* actif dans le métabolisme.

Conclusions.

1° Alors que l'adsorption d'un savon par une micelle diastasique telle que la pancréatine ou l'uréase reste sans effet sur son activité, la fixation d'un acide gras sur la pepsine et l'uréase modifie notablement cette activité.

2° Pour des concentrations en pepsine et en uréase, fortes par rapport à la concentration en acides gras, le pouvoir inhibiteur d'un acide gras croit avec sa concentration et s'accuse beaucoup plus avec les acides non saturés.

3° Dans le cas de l'uréase, deux facteurs interviennent pour modifier l'activité diastasique de l'enzyme ainsi enrobée : force d'étalement de l'acide et section du film périmicellaire. La pression osmotique du liquide extérieur conditionne alors l'effet résultant. La cinétique de la réaction ne permet plus d'appliquer la formule de Van Slyke.

(1) Les flèches n'indiquant plus ici un déplacement d'équilibre, mais le passage d'un constituant à l'autre.

4° Il est possible de supposer que l'équilibre ou le déséquilibre des jeux diastasiques *in vivo* est en partie sous la dépendance de couples diastasiques analogues.

(*Laboratoire de Chimie biologique et médicale de la Faculté de Médecine de Lyon*, Professeur L. Hugounenq).

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[Contribution from the Department of Chemistry, National Taiwan University]

Polyamide Impregnated Paper Chromatography. II.* Aliphatic Acids and Amides

By

KUNG-TSUNG WANG (王光璁)

Previously developed polyamide impregnated paper chromatography was applied to the aliphatic acids and amides. The Rf values were tabulated.

In the previous paper[1] we described a fine separation of phenols with good reproducibility of Rf values by the use of newly deviced polyamide impregnated paper chromatography. These results led us to expect further application of this method to other substances. The pioneer paper of Carelli, Liquori, and Mele[2] described the frontal analysis and displacement chromatography of propionic acid, butyric acid and caproic acid by polyhexamethyene adipamide powder column and showed a promising result for the separation of these substances. They found that the sorption decreased with the increasing number of carbon atoms. Although the application of polyamide column chromatography to the separation of polyphenolic substances,[3] 2,4-dinitrophenyl amino acids[4] and hydroxy derivatives of anthracene[5] has been reported, further study in its application to other substances is still to be made.

In the present work the polyamide impregnated paper chromatography was applied to the normal aliphatic acids and amides. There results and some physical conatants are listed in the Table

*The paper with the same title that appeared in this Journal [Series II, 673 (1959)] is regarded as the first part of the series.

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ose of the amides were develop
and then spraying with 1% alco
ric chloride to give light red spot. The norm
less than ten carbon atoms showed good separ
the tailing spots. The difference of the Rf
acids was about 0.08 except for formic acid a
trend of the increase of Rf values with increa
is the same as that of the boiling points of n
The difference of Rf between formic acid
anomalously large. This is in accord with th
exists in the case of other physical constants
to be limited to the acids with fewer than
cause the acids from C_{16} up have the same I

The molecular attraction of amide is
corresponding acid as is reflected in the chr
ethyl acetate, formamide moved only a little
was the limit (Rf 0.86). With amides highe
appeared on the solvent front. By the use o
acetamide and propionamide did not move.
a little (Rf 0.05) and myristamide was a limit
tion of the spots on the chromatogram was
the use of a proper solvent, we can estimate
atoms presented in the amide.

Table I. Acids

	b.p.[6]	pKa[6]
Formic	100.7°	3.77
Acetic	118.1°	4.76
Propionic	141.1°	4.88
Butyric	163.5°	4.82
Valeric	187°	4.86
Caproic	205°	4.88

ent of Chemistry, National Taiwan University

nated Paper Chromatography. tic Acids and Amides

By

ING WANG (王[illegible])

polyamide impregnated paper chroma- e aliphatic acids and amides. The Rf

escribed a fine separation of bility of Rf values by the use of newly ated paper chromatography. These rther application of this method to neer paper of Carelli, Liquori, and analysis and displacement chromato- yric acid and caproic acid by polyhex- column and showed a promising result bstances. They found that the sorp- reasing number of carbon atoms. olyamide column chromatography to substances,[3] 2,4-dinitrophenyl amino s of anthracene[5] has been reported, n to other substances is still to be

olyamide impregnated paper chro- normal aliphatic acids and amides. l conatants are listed in the Table

that appeared in this Journal (Series II, art of the series.

I, and II. The spots of the acids were developed by bromophenol blue,[6] and those of the amides were developed by conversion to hydroxamate and then spraying with 1% alcoholic solution of ferric chloride to give light red spot. The normal aliphatic acid with less than ten carbon atoms showed good separation although giving the tailing spots. The difference of the Rf value for neighboring acids was about 0.08 except for formic acid and acetic acid. The trend of the increase of Rf values with increasing molecular weight is the same as that of the boiling points of normal aliphatic acids. The difference of Rf between formic acid and acetic acid was anomalously large. This is in accord with the similar situation that exists in the case of other physical constants. The serption seems to be limited to the acids with fewer than ten carbon atoms, because the acids from C_{10} up have the same Rf values.

The molecular attraction of amide is far stronger than the corresponding acid as is reflected in the chromatogram. By using ethyl acetate, formamide moved only a little (Rf 0.14) and octamide was the limit (Rf 0.86). With amides higher than C_8, the spots appeared on the solvent front. By the use of benzene, formamide acetamide and propionamide did not move. n-Butyramide moved a little (Rf 0.05) and myristamide was a limit (Rf 0.90). The separation of the spots on the chromatogram was quite satisfactory. By the use of a proper solvent, we can estimate the number of carbon atoms presented in the amide.

Table I. Acids

	b.p.[6]	pKa[7]	Rf (cyclohexane as a solvant)
Formic	100.7°	3.77	0.01
Acetic	118.1°	4.76	0.19
Propionic	141.1°	4.88	0.27
Butyric	163.5°	4.82	0.35
Valeric	187°	4.86	0.45
Caproic	205°	4.88	0.51

Heptylic	223.5°	4.89	0.63
Caprylic	237.5°	4.90	0.71
Capric	268–70°		0.83
Lauric	225° (100 mm)		0.87
Mytistic	250.5° (100 mm)		0.87
Palmitic	339–55° (decomp.)		0.87
Stearic	383°		0.87

Table II. Amides

Solvents	Rf Values	
	Benzene	Ethyl acetate
Foramide	0	0.14
Acetamide	0	0.20
Propionamide	0	0.31
n-Butyramide	0.05	0.40
n-Va-eramide	0.14	0.52
n-Caproamide	0.22	0.64
n-Heptamide	0.30	0.72
n-Octamide	0.50	0.86
n-Capriamide	0.62	f*)
Lauriamide	0.77	f
Myristamide	0.90	f
Palmitamide	0.90	f
Stearamide	0.90	f

*) f means spots on solvents front

Experimental

Paper. The paper was twice impregnated[1] with 5% polycapro lactam formic acid solution.

Samples. The acids at hand were of varying grades, so they were mostly purified before use. The physical constants were listed. The amides were prepared by the usual method except formic and acetic acid which were commercial reagents. All the melting points were not corrected.

Acids

Formic	b.p.	106–106.5° (azeotropic)	Take
Acetic	b.p.	117–117.5°	Take
Propionic	b.p.	139–139.5°	
Butyric	b.p.	160.5–161°	
Valeric	b.p.	185.5–186°	
Caproic	b.p.	203–203.5	
Heptylic		Kahlbaum	
Caprylic		Kahlbaum	
Capric	m.p.	30–30.5	
Lauric		Kahlbaum	
Myristic	m.p.	53–53.5°	
Palmitic	m.p.	62–62.5°	
Stearic	m.p.	69–69.5°	

The sample was dissolved in methanol The quantity applied was from 8 μ to 12 μ.

Solvents Cyclohexane, Benzene, and ethyl distilled.

Chromatography. The method used was th described[1].

Colour development.

Acids. The acids were developed by br tor to give yellow spots.[3]

Amides. The saturated methanolic solu hydrochloride was sprayed on the dried chr at 100° in an air oven for five minutes, the with 1% ethanolic solution of ferric chlorid ed immediately, because the colour would minutes.

Acknowledgment

The author wishes to express his heartf

	4.80	0.63
	4.90	0.71
		0.83
m)		0.87
mm)		0.87
comp.)		0.87
		0.87

Table II. Amides

Rf Values	
Benzene	Ethyl acetate
0	0.11
[illegible]	0.20
[illegible]	0.31
0.05	0.40
0.14	0.52
0.22	0.64
0.30	0.72
0.50	0.86
0.62	f*)
0.77	f
0.90	f
0.90	f
0.90	f

nts front

Experimental

twice impregnated[1] with 5% polycapro

hand were of varying grades, so the
use. The physical constants were listed
by the usual method except formic and
mmercial reagents. All the melting

		Acids	Amides
Formic	b.p.	106-106.5° (azeotropic)	Takeda brand. E.P. reagent
Acetic	b.p.	117–117.5°	Takeda brand. E.P. reagent
Propionic	b.p.	139–139.5°	75–76°
Butyric	b.p.	160.5–161°	113–114°
Valeric	b.p.	185.5–186°	103–104°
Caproic	b.p.	203–203.5	98–99°
Heptylic		Kahlbaum	94–95°
Caprylic		Kahlbaum	105–106°
Capric	m.p.	30–30.5	97–98°
Lauric		Kahlbaum	95–96°
Myristic	m.p.	53–53.5°	100–100.5°
Palimtic	m.p.	62–62.5°	103–104°
Stearic	m.p.	69–69.5°	107–108°

The sample was dissolved in methanol to make 1% solution. The quantity applied was from 8 μ to 12 μ.

Solvents Cyclohexane, Benzene, and ethyl acetate were twice distilled.

Chromatography. The method used was the same as previously described[1].

Colour development.

Acids. The acids were developed by bromophenol blue indicator to give yellow spots.[5]

Amides. The saturated methanolic solution of hydroxylamine hydrochloride was sprayed on the dried chromatogram and heated at 100° in an air oven for five minutes, then followed by spraying with 1% ethanolic solution of ferric chloride. The spot was marked immediately, because the colour would disappear after a few minutes.

Acknowledgment

The author wishes to express his heartful thanks to Prof. Y. T.

Lin for his encouragement and suggestion. He is also indebted t
Mr. S. C. Kao for his technical assistance.

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tribution from the Department of Chemistry, National T

Identification of the Products from the F o- and p-Iodophenyl Acetate wi Aluminium Chloride

By

CHIN-TE CHANG (張錦德) and FA-CHING CHE

The products, (I) m.p. 69-71° and (II) m.p. 171-17 from the reaction of *o*- and *p*- iodophenyl acetate wit aluminium chloride were elucidated as 2,4-diiodophen diiodo-4-hydroxyacetophenone respectively. Suitable the preparation of diiodophenol and diiodohydroxya were also described.

In the course of the attempted synthesis of vones in the usual way,[1] we investigated the F al of *o*- and *p*-iodophenyl acetate, expecting to obt and 3-iodoacetophenone (unknown) as starting ther synthesis. Our first attempts were unsucc ying the reaction temperature, time, solvent, and a small amount of colourless crystal (I), m.p. the Fries-rearrangement of *o*- and *p*-iodophe the Friedel-Craft reaction of *p*-iodophenol. Th p. 151-156°, after recrystallization raised t ed by the analogous treatment of *o*-iodophen The synthesis of 2-hydroxy-3- and 5-iodoace accomplished by taking the routes* as shown in quations:

* The results will be reported elsewhere in detail. have been brought forward.[5,6,7]

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Fatty acids and pronethalol-induced reversal of saline diuresis

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WAUGH, WILLIAM H., AND BARBARA K. URBAITIS. *Fatty acids and pronethalol-induced reversal of saline diuresis.* Am. J. Physiol. 219(3): 626–635. 1970.—Dogs were volume expanded by means of isotonic sodium infusions, which were sustained at 0.4–0.5 ml/kg per min. Unilateral renal arterial infusions of the lipokinetic agent, pronethalol, from near-threshold doses for natriuretic reversal to doses which caused sizable reversal of the saline diuresis, failed to reveal a significantly greater antinatriuretic effect in the ipsilateral kidney. Unilateral arterial infusions of palmitate and octanoate failed to suppress the sodium diuresis. However, octanoate given into one renal artery inhibited ipsilaterally tubular *p*-aminohippurate clearance (C_{PAH}); pronethalol-induced marked elevations in endogenous plasma FFA level were not associated with decreases in tubular C_{PAH}. Infusions of nicotinate as antilipolytic agent, but not injection of butoxamine or *N*-isopropylmethoxamine, dissociated the lipolytic effect of pronethalol from its reversal of natriuresis. In these volume-expanded dogs, pretreatment with phenoxybenzamine uncovered a mild but significant hypotensive effect of pronethalol during its natriuretic suppression, which occurred without measurable change in glomerular filtration rate or C_{PAH}. The findings suggest that the antinatriuretic effect of pronethalol may be related causally to its systemic beta-adrenergic vascular or vasodilator action rather than its lipokinetic effect and that octanoate anion is capable of in vivo competition with PAH for the tubular organic acid transport system.

extracellular volume control; renal sodium excretion; nicotinic acid; adrenergic blocking agents; lipolytic agents; PAH-organic acid transport system

THE MAJOR REGULATING MECHANISMS for renal sodium excretion exclusive of changes in glomerular filtration rate or adrenal mineralocorticoid hormonal activity are presently unclear (13, 23). Recently, it was shown that injection of the lipolytic or lipokinetic beta-adrenergic receptor blocking agents, pronethalol and dichloroisoproterenol (DCI), markedly inhibited the development of sodium diuresis with moderate saline loads and that this inhibition correlated with induced elevations in the levels of endogenous free fatty acids (FFA) in arterial plasma (38). The findings suggested that metabolic inhibition of active tubular sodium transport might be a determinant of renal sodium excretion during extracellular volume expansion. However, the study did not determine whether an aromatic amine, such as pronethalol, which directly mobilizes FFA by increasing lysis of triglycerides, stimulates tubular sodium transfer during saline diuresis by a direct or indirect renal effect.

To attempt to answer this question, the following experiments were performed during saline diuresis. These experiments included local intra-arterial infusions of exogenous FFA and of pronethalol into one kidney and focused upon the reversal or inhibition of natriuresis with continuing volume expansion.

METHODS

Mongrel dogs, both male and female, between 12 and 20 kg body wt, were used for these experiments. The afternoon before the experiment each dog was given 5 units of vasopressin tannate in oil and 10 mg of deoxycorticosterone acetate (DOCA) in oil intramuscularly. Food was then withheld but water allowed ad libitum. On the morning of the experiment, 15 mg of morphine sulfate were given subcutaneously, and about 1 hr later the dog was anesthetized with intravenous sodium pentobarbital, 24 mg/kg. Five milligrams of DOCA in oil were given intramuscularly at this time. Supplemental pentobarbital was given during the course of the experiment to maintain a plane of light surgical anesthesia.

Endotracheal intubation was then performed. The left jugular vein was catheterized, and a priming solution containing 700 mg of creatinine and 200 mg of para-aminohippurate (PAH) was administered. Following this, a loading solution containing 120 mM NaCl, 24 mM $NaHCO_3$, 3 or 4 mM KCl, and 0.5 mg creatinine per milliliter and 0.25 mg PAH per milliliter was delivered intravenously at 1 ml/kg for 60 min. At the end of this time, a solution containing identical concentrations of electrolytes, but 2.5 or 2 times the concentrations of creatinine and PAH, was given intravenously at the rate of 0.4 or 0.5 ml/kg per min for the duration of the experiment. These infusion rates were adequate to maintain relatively constant plasma levels of creatinine and of PAH near 8–12 and 1–2.5 mg/100 ml, respectively.

During the 60-min time for the administration of the loading solution, the necessary surgical procedures were performed. The bladder was exposed via a suprapubic incision, and the ureters catheterized proximal to their entry into the bladder. Urine was collected separately from each kidney into graduated cylinders. In some experiments both ureters were catheterized, but the urine from each kidney was pooled together and collected as a single sample. One femoral artery was catheterized with fine plastic tubing for arterial blood sampling and for the measurement of mean arterial blood pressure and, in some cases, heart rate. Mean blood pressures were recorded from a strain-gauge

Cap. Acid

manometer (Statham P23AA transducer) by use of an oscillographic recorder (Electronics for Medicine, Inc., White Plains, N.Y.). Heart rate was recorded similarly on paper from an Electronics for Medicine tachometer amplifier, connected to a pressure amplifier and another strain-gauge manometer, set in series to detect femoral arterial pulses.

In those experiments in which FFA or pronethalol was administered to one kidney, the renal artery supplying that kidney was previously exposed via a flank incision. A no. 23-gauge curved needle attached to a length of PE-20 tubing was placed into the main renal artery well before its first bifurcation. A constant infusion of isotonic saline was delivered into the artery at 0.97 ml/min to maintain patency of the needle, before and after FFA or pronethalol infusion. In those experiments in which a drug was given intravenously, it was delivered into the jugular vein through the same tubing that carried the continuous saline infusion.

About 1 hr or so after institution of the constant saline infusions at 0.4 or 0.5 ml/kg per min and when diuresis was essentially stabilized, urine samples were collected for two or three 10-min control periods. Arterial blood samples were collected at the midpoint of each collection period. Following the control collections, the agent under study (FFA or pronethalol) was infused. During and after the infusion of test agent a number of 10-min urine collections were obtained serially.

In experiments employing nicotinic acid, the sodium salt of nicotinic acid was used; a priming dose of 515 μmoles/kg (mg/kg) was given intravenously 40–70 min before the start of the control periods and sodium nicotinate, 150 mM, was continued as a constant intravenous infusion of 1.23 ml/min or 184 μmoles/min for the duration of the experiment. In those cases in which phenoxybenzamine (Dibenzyline—Smith, Kline, and French Laboratories) was under study, the drug, as the hydrochloride salt dissolved in 150 mM sodium chloride solution, was given intravenously in a dose of 2 or 5 mg/kg, 30–40 min before the start of the control collection periods. Pronethalol·HCl (Alderlin, Ayerst Laboratories; mol wt 266) and butoxamine·HCl and N-isopropylmethoxamine·HCl (Burroughs Wellcome & Co.) were given dissolved in isotonic sodium chloride solution. Sodium palmitate solutions of 18–19 mM, in 15 g serum albumin/100 ml of solution containing 150 mM sodium chloride, were prepared from palmitic acid according to a slight modification of the method of Evans and Mueller (14). Sodium octanoate solutions of 40–200 mM, in 5 g serum albumin/100 ml of solution of 150–200 mM total sodium concentration, were prepared with NaOH neutralization of added octanoic acid. These solutions were infused at rates between 0.38 and 1.94 ml/min for 25 min. The albumin solutions were made from bovine or canine serum albumin, fraction V powder, obtained from Pentex Inc. In experiments with infusions of palmitate- and octanoate-albumin solutions, appropriate intra-arterial infusions of 5–15 g serum albumin/100 ml of solution of 150 mM sodium chloride were made during control urine collection periods.

Analytical procedures for plasma and urine creatinine, PAH, and Na, and for plasma FFA, total protein, and K were performed as previously described (38). Hematocrits of arterial blood samples were determined in capillary tubes by use of an International microcapillary centrifuge and an International microcapillary reader. Renal plasma clearances of creatinine (C_{Cr}) and PAH (C_{PAH}) were used as indices of glomerular filtration rate (GFR) and effective renal plasma flow, respectively. Filtered loads of sodium were calculated without use of a Donnan factor.

RESULTS

Effect of unilateral renal arterial infusions of palmitate and octanoate during saline diuresis. The effects of local arterial infusions of varying amounts of palmitate and octanoate on sodium diuresis, on C_{Cr}, and on local arterial increments in plasma FFA level are summarized in Table 1. The effects of local palmitate and octanoate infusions on total C_{PAH}, on tubular clearances of PAH, and on the calculated molar concentration ratios of exogenous FFA/PAH in the plasma flowing into the kidney unilaterally are shown in Table 2. The results are expressed as changes, using as control values both the values found immediately before renal arterial infusions of palmitate or octanoate and the simultaneously determined functional values found in the contralateral or opposite kidney undergoing sodium diuresis. In the tabulated results, the local increment in FFA of the arterial plasma of the infused kidney was estimated from the infusion rate of FFA and the total renal plasma flow, calculated from the concurrently measured C_{PAH} or, when depressed unilaterally, from the antecedent C_{PAH} of that kidney. This was done by assuming an average PAH renal plasma extraction ratio of 0.80. Tubular C_{PAH} was determined by subtraction of the value for clearance of PAH by glomerular filtration (C_{Cr}) from the value for total renal plasma clearance of PAH (C_{PAH}). No correction term for protein binding of PAH was applied to the C_{Cr} values for estimation of glomerular clearance of PAH. This appeared justifiable, since binding of PAH to plasma proteins appears negligible (near 1%) in the dog. This follows from the reports that the product of the unbound fraction of PAH in plasma water and the plasma water content averages 0.92 in the dog (34) and that dog plasma water content averages 0.93 (1). Tubular clearance of PAH represents the virtual renal plasma volume flow which is completely cleared of PAH by tubular secretion. Changes in tubular C_{PAH} values, at low plasma PAH levels, result from changes in tubular secretion of PAH and/or in peritubular plasma flow.

As shown in Tables 1 and 2, in three experiments employing renal arterial infusions of palmitate in doses between 19 and 37 μmoles/min, there was not a significant ipsilateral difference or change in the magnitude of the natriuresis (+8 ± 19 μEq/min change), C_{Cr} (0 ± 0.6 ml/min change), total C_{PAH} (+4.2 ± 4.8% change), or tubular C_{PAH} (+6.0 ± 4.8% change) (mean ± SEM). The palmitate infusions elevated the local arterial plasma concentration of FFA an average of 0.23 mM, and the concentration of exogenous palmitate exceeded the plasma molar concentration of PAH by approximately 3 times. Larger amounts of palmitate were not given because of the large volume rates of intra-arterial infusion of 15% albumin solutions required for this purpose (>2.0 ml/min).

Renal arterial infusion of octanoate at a similar molar

TABLE 1. *Sodium excretions and creatinine clearances with unilateral renal arterial infusions of palmitate and octanoate during saline diuresis*

Dose of FFA, μmoles/min	Kidney	$U_{Na}V$, μEq/min			C_{Cr}, ml/min			Art P_{FFA}, mM
		Before	During, change	Change, inf-opp	Before	During, change	Change, inf-opp	During
Palmitate, 19-37	Inf	357 ± 38	−10 ± 50	+8 ± 19[a]	33 ± 1.3	−1 ± 0.9	0 ± 0.6	+0.23 ± 0.03[d]
	Opp	334 ± 34	−21 ± 33		32 ± 2.1	−1 ± 0.9		0.50 ± 0.08
Octanoate, 39	Inf	334	−21	−36	33	+1	0	+0.36[d]
	Opp	318	+15		40	+1		0.22
Octanoate, 86	Inf	129	+7	+1	31	0	+1	+0.52[d]
	Opp	141	−6		33	−1		0.42
Octanoate, 102-117	Inf	290 ± 14	+66 ± 52	+31 ± 23[b]	33 ± 2.7	+3 ± 1.2	+4 ± 0.9[c]	+0.91 ± 0.01[d]
	Opp	285 ± 4	+35 ± 34		34 ± 3.5	−1 ± 1.2		0.37 ± 0.07
Octanoate, 194	Inf	437	+30	+38	40	−1	+6	+1.81[d]
	Opp	496	−8		46	−7		0.30

Values are means ± SEM of three experiments or mean values in individual experiments. Inf = ipsilaterally infused; opp = contralateral kidney; before = values found over 20-min interval just before the fatty acid infusion; during = values found over last 20 min of fatty acid infusion. [a] $P > 0.5$. [b] $P < 0.3$. [c] $P > 0.05$, from paired t test ($n = 3$). [d] Local plasma increment, estimated from the infusion rate of palmitate or octanoate and the RPF, calculated (by assuming a PAH extraction ratio of 0.80) from the concurrent C_{PAH} or, when depressed unilaterally, from antecedent C_{PAH}.

TABLE 2. *Total renal and tubular clearances of PAH with unilateral renal arterial infusions of palmitate and octanoate during saline diuresis*

Dose of FFA, μmoles/min	Kidney	Total C_{PAH}			Tubular C_{PAH}			Inf Plasma Molar Ratio (Extra FFA/PAH)
		Before, ml/min	During, % change	% Change, inf-opp	Before, ml/min	During, % change	% Change, inf-opp	
Palmitate, 19-37	Inf	101 ± 6	+6.7 ± 4.0	+4.2 ± 4.8[a]	68 ± 5	+10.6 ± 3.0	+6.0 ± 4.8[a]	3.1 ± 0.3
	Opp	99 ± 6	+2.5 ± 2.2		66 ± 4	+4.6 ± 3.3		
Octanoate, 39	Inf	74	+16	+3	41	+27	+3	4.5
	Opp	90	+13		50	+24		
Octanoate, 86	Inf	133	−27.0	−24.7	102	−35	−33	8.1
	Opp	128	−2.3		95	−2		
Octanoate, 102-117	Inf	98 ± 4	−25.1 ± 5.1	−24.2 ± 3.0[b]	65 ± 3	−42 ± 5.3	−43 ± 4.7[b]	13.4 ± 0.4
	Opp	98 ± 6	−0.9 ± 2.2		64 ± 5	−1 ± 4.0		
Octanoate, 194	Inf	86	−29.1	−25.9	46	−52	−60	44.0
	Opp	95	−3.2		49	+8		

Values are means ± SEM of three experiments or mean values in individual experiments. Inf, opp, before, and during represent same items as in Table 1. Arterial plasma PAH levels ranged between 0.8 and 1.7 mg/100 ml. Infused plasma molar ratios were calculated from the local plasma increment in palmitate or octanoate, as shown in Table 1. [a] $P > 0.3$, from paired t test ($n = 3$). [b] $P < 0.02$. Dog weight: 16.9 ± 1.7 kg (mean ± SD, $n = 7$).

rate (e.g., 39 μmoles/min) failed similarly to influence any of the above parameters during saline diuresis. In addition, renal arterial infusion of octanoate in slightly greater amounts, which elevated local arterial plasma levels of octanoate by 0.5–1.8 mM, failed also to inhibit significantly the natriuresis of continuing volume expansion ipsilaterally (e.g., change of +31 ± 23 μEq Na/min, mean ± SEM, $P > 0.3$) or contralaterally. With such dose infusions of octanoate, mean C_{Cr} was slightly or equivocally increased in the kidney receiving the octanoate, as shown in Table 1. On the other hand, these larger renal arterial infusions of octanoate (at 86–194 μmoles/min) reduced total C_{PAH} ipsilaterally, and this reduction was mediated by a large decrease (by 33–60%) in the tubular clearance moiety of renal C_{PAH}, as shown in Table 2, without decrease in C_{Cr} or GFR.

The inhibition of tubular C_{PAH}, without inhibition of saline diuresis or C_{Cr}, was related posologically to the rate of octanoate infusion and to the local arterial plasma concentration of octanoate (see Table 1). More particularly, the ipsilateral depression of tubular C_{PAH} induced by octanoate infusion seemed related to the simultaneous molar concentration ratio of octanoate/PAH in the delivered arterial plasma. This latter relationship is evident in Table 2 and in graphical form in Fig. 1.

Effect of unilateral renal arterial infusions of antinatriuretic amounts of pronethalol during saline diuresis. The administration of pronethalol into one renal artery (or intravenously) at a constant rate for 20–30 min to total dosage of 2.7–4.0 μmoles/kg resulted in appreciable inhibition of saline diuresis (e.g., Table 5). With these dosages of pronethalol,

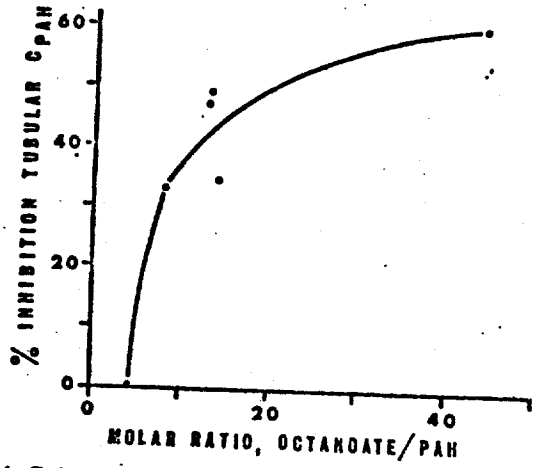

FIG. 1. Relationship of plasma molar ratio of octanoate/PAH to percentage inhibition of tubular clearance of PAH at non-Tm plasma levels of PAH in vivo. Detectable inhibition of tubular C_{PAH} and, presumably, tubular excretion of PAH occurred at an estimated renal arterial plasma concentration of 0.36 mM octanoate (molar ratio of 8.1), but not at 0.23 mM (molar ratio of 4.5).

given intra-arterially or else intravenously, as illustrated serially in Fig. 2 with intravenous administration after phenoxybenzamine (vide infra), the natriuretic inhibition began frequently with the first 10 min of pronethalol infusion, and the inhibition of natriuresis was maximal almost invariably during the first 10-min interval after stoppage of pronethalol infusion. This reversal of sodium diuresis was generally moderately less than maximal during the last min of pronethalol infusion and slightly less than maximal during the second 10-min interval after cessation of pronethalol infusion. However, when the constant infusions of pronethalol were prolonged for 40–50 min (to similar total dosage), the inhibition of saline diuresis was usually somewhat greater during the last 10 min of pronethalol infusion than in the second 10-min interval after stoppage of pronethalol administration. These cumulative and temporal differences in response are consistent with the pharmacologic and metabolic mean half-lives of pronethalol, which are about 40–60 min (4).

In addition, with unilateral arterial infusion of pronethalol into one kidney, the time of onset of the reduction of saline diuresis was generally similar in the kidney locally infused with pronethalol to that in the contralateral kidney. Therefore, to quantitate the inhibition of sodium diuresis induced by infusions of pronethalol, the average of the values found during the first two 10-min periods after cessation of pronethalol administration was used when the drug infusions were for 20–30 min, and the average of the values found during the last 10-min period during infusion and the first 10 min period after stoppage of pronethalol infusion was employed when infusions were performed for 40–50 min. These values were compared to the mean control parameters found in the two 10-min periods immediately before the beginning of the pronethalol infusions.

Differential renal functional studies during saline diuresis performed in six experiments with unilateral arterial ions of pronethalol ranging from 2.3 to 4.0 μmoles/kg, given over 20–40 min. These results are summarized in Table 3. In the kidney locally infused with pronethalol, mean sodium excretion was reduced by 56 ± 14% (mean ± SD, $P < 0.001$) from a control rate of 300 ± 109 μEq/min (mean ± SD), whereas the contralateral excretion of sodium was reduced by 48 ± 10% ($P < 0.001$) from its average control rate of 287 ± 90 μEq/min. The mean difference between the ipsilateral and contralateral kidneys in the pronethalol-induced percentage inhibition of the natriuresis was not significant (−8.1 ± 5.4%, mean ± SEM, $P > 0.1$). This natriuretic suppression was characterized by a similar degree of pronethalol-induced inhibition of fractional sodium excretion (percentage of filtered sodium excreted) and of diuretic urine flow, as also shown in Table 3. Also, neither the percentage reversal of the high fractional sodium excretion of around 4–8.5%, nor the percentage reversal of the diuretic urine flows, was significantly greater in the kidney into whose main artery the pronethalol was administered than in the contralateral kidney ($P > 0.1$ and $P > 0.5$, respectively). Table 3 also shows that sizable reversal of sodium diuresis by pronethalol infusion took place with no measurably significant change in mean C_{Cr} and C_{PAH} in either ipsilateral or contralateral kidney.

In four other experiments, differential renal studies during saline diuresis were performed with use of smaller amounts of pronethalol infused into a renal artery unilaterally. These

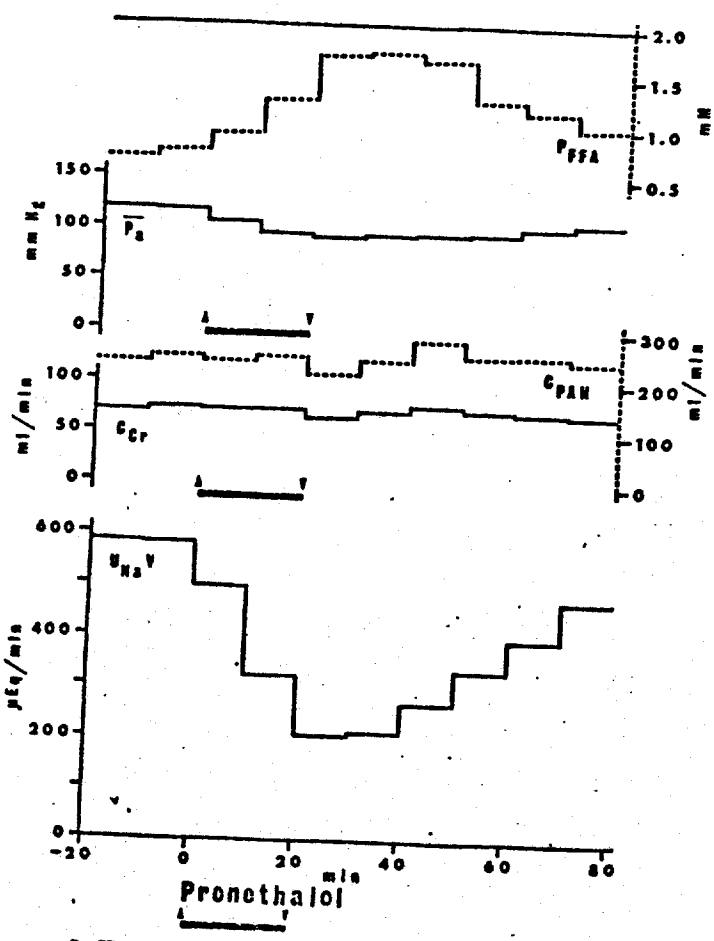

FIG. 2. Temporal effects of intravenous pronethalol, 4 μmoles/kg, during alpha-adrenergic blockade on sodium excretion, C_{Cr}, C_{PAH}, mean arterial blood pressure, and P_{FFA} in a natriuretic dog receiving saline. 540 μmoles of isotonic sodium salt/min were infused continuously, after prior loading with 60 ml/kg. Phenoxybenzamine, 5 mg/kg, and N-isopropyl-methoxamine, 7.5 mg/kg, were injected intravenously 30–45 min before beginning of 20-min pronethalol infusion. Dog weight = 15 kg.

TABLE 3. *Differential studies with unilateral renal arterial infusion during saline diuresis*

Mean Values	Kidney	Controls Before	Paired Change	Paired Percent Change	Difference, Percent Change (inf − opp)
$U_{Na}V$, μEq/min	Inf	300±109	−159±52	−56 ±14%[a]	−8.1 ±5.04%[a]
	Opp	287±90	−132±25	−48 ±10%[a]	
F_{Na} excreted, %	Inf	6.4±2.4	−3.4 ±0.91	−56 ±16%[a]	−9.2 ±5.24%[a]
	Opp	5.9±1.8	−2.6 ±0.66	−46 ±11%[a]	
V, ml/min	Inf	1.96 ±0.97	−1.11 ±0.56	−60 ±16%[a]	−11 ±5.34%[d]
	Opp	1.82 ±0.89	−0.85 ±0.52	−49 ±18%[b]	
C_{Cr}, ml/min	Inf	32±5.4	−0.2 ±2.4		
	Opp	33±5.0	−0.4 ±1.7		
C_{PAH}, ml/min	Inf	87±16	+1.8±16		
	Opp	88±19	+3.3 ±8.8		

Values are mean ± SD, except for the inf minus opp differences in percentage change, the values of which are means ± SEM. Infusion = 2.3–4.0 μmoles pronethalol/kg. Inf = ipsilaterally infused kidney; opp = contralateral kidney; controls = values found over 20-min interval just before pronethalol infusion for 20–40 min into one renal artery (mean total dosage of 3.0 μmoles/kg); paired change = difference between control values and values found over first 20 min after stoppage of pronethalol infusion. [a] $P < 0.001$. [b] $P < 0.01$. [c] $P > 0.1$ from paired t test. [d] $P > 0.05$. $n = 6$ for all parameters. Dog weight: 16.6 ± 2.8 kg (mean ± SD).

doses totaled 1.0–1.3 μmoles of pronethalol per kilogram and they were given over an interval of 30–50 min; they were close to the threshold amounts for inducing any reversal of the saline diuresis. Nevertheless, these amounts of pronethalol produced some lipokinesis, for plasma free fatty acid levels rose from 0.27 ± 0.04 (mean ± SEM) to 0.74 ± 0.13 mM (paired increment of +0.48 ± 0.11 mM, $P < 0.05$). Table 4 summarizes these differential renal functional results. The average renal sodium excretion from a control natriuretic rate of 347 ± 88 μEq/min on the contralateral side changed merely by −18 ± 24% (mean ± SD, $P > 0.1$); however, mean sodium excretion from a control natriuretic rate of 390 ± 100 μEq/min in the kidney receiving pronethalol by direct arterial infusion declined by a similar amount (−16 ± 17%, $P > 0.1$), and the mean difference in percentage change of +1.9 ± 4.8% (mean ± SEM) between the two sides was not significant ($P > 0.5$). Similarly, the percentage of filtered sodium excreted, from values of 8.4 ± 2.0% ipsilaterally (mean ± SD) and values of 7.5 ± 2.4%, contralaterally, were not inhibited more in the kidneys into which the pronethalol was infused locally; the mean difference (ipsilateral-contralateral) in percent change of +1.2 ± 3.1% (mean ± SEM) was not significantly different from zero ($P > 0.5$). Mean C_{Cr} and C_{PAH}, also, did not change significantly.

In order to quantitate the total changes in sodium diuresis found in both kidneys when renal arterial infusions of appreciable antinatriuretic amounts of pronethalol were administered, the values in both kidneys were added or averaged in each of five experiments with pronethalol infusions totaling 2.7–4.0 μmoles per kilogram, given over 20–30 min. These results are summarized in Table 5, along with the associated mean plasma concentrations of free fatty acid, potassium, and protein. From control sodium excretions of 631 ± 98 μEq/min, from control fractional sodium excretions of 6.5 ± 0.93%, and from control urine flows of 4.10 ± 0.85 ml/min (mean ± SEM), pronethalol administration resulted in average percentage reductions of −51 ± 5.3% ($P < 0.001$), −50 ± 5.9% ($P < 0.01$), and −55 ± 8.3% ($P < 0.01$), respectively. The C_{Cr} and C_{PAH} did not measurably change with this pronethalol inhibition of saline diuresis, for the mean changes of −0.7 ± 1.7 and +6 ± 13 ml/min, respectively, were not significant ($P > 0.5$). Mean plasma potassium level fell slightly by 0.26 ± 0.093 mM ($P < 0.05$), whereas the low plasma protein level of 4.1 ± 0.34 g/100 ml (mean ± SEM) induced by the volume expansion with saline did not appreciably change ($P > 0.5$) during the marked reversal of the sodium diuresis by pronethalol. On the other hand, these doses of pronethalol (mean of 3.2 μmoles/kg) resulted in marked fatty acid mobilization during the natriuretic inhibition; mean arterial plasma FFA level rose by 0.78 ± 0.058 mM (mean ± SEM, $P < 0.001$) from an average value of 0.37 ± 0.15 mM just before pronethalol infusion. In only one of these five experi-

TABLE 4. *Differential studies with unilateral renal arterial infusion during saline diuresis*

Mean Values	Kidney	Controls Before	Paired Change	Paired Percent Change	Difference, Percent Change (inf − opp)
$U_{Na}V$, μEq/min	Inf	390±100	−70±85	−16 ±17%[a]	+1.9 ±4.8%[b]
	Opp	347±88	−71 ±104	−18 ±24%[a]	
F_{Na} excreted, %	Inf	8.4±2.0	−1.6 ±1.6	−18 ±15%[a]	+1.2 ±3.17[b]
	Opp	7.5±2.4	−1.7 ±2.4	−19 ±24%[a]	
C_{Cr}, ml/min	Inf	32±3.3	+0.8 ±4.4		
	Opp	32±3.0	+0.7 ±1.0		
C_{PAH}, ml/min	Inf	81±14	−0.9 ±3.9		
	Opp	85±15	−4.4 ±5.8		

Values are means ± SD, except for the inf minus opp differences in percentage change, where values are means ± SEM. Infusion = 1.0–1.3 μmoles pronethalol/kg. Inf = ipsilaterally infused kidney; opp = contralateral kidney; controls = values found over 20-min period just before pronethalol infusion for 30–50 min into one renal artery (mean total dose of 1.2 μmoles/kg); paired change = difference between control values and values found over first 20-min interval after stoppage of 30-min infusion of pronethalol (2 exp) or values found over last 10 min of infusion and first 10 min after stoppage of 40- to 50-min infusion of pronethalol (2 exp). [a] $P > 0.1$. [b] $P > 0.5$, from paired t test. Dog weight: 16.4±1.8 kg (mean±SD).

TABLE 5. *Reversal of saline diuresis by short-term pronethalol infusion during continuous infusion of saline intravenously*

Mean Values	Controls Before	After Pronethalol	Paired Change	Paired Percentage Change
$U_{Na}V$, μEq/min	631±98	323±79	−307 ±28[a]	−51 ±5.3%[a]
F_{Na} excreted, %	6.5±0.93	3.4 ±0.76	−3.1 ±0.27[b]	−50 ±5.9%[b]
V, ml/min	4.10 ±0.85	1.98 ±0.57	−2.11 ±0.48[c]	−55 ±8.3%[b]
P_{FFA}, mM	0.37 ±0.15	1.14 ±0.15	+0.78 ±0.058[a]	+450 ±227%[e]
P_K, mM	3.5±0.13	3.2±0.09	−0.26 ±0.093[c]	−7.0 ±2.4%[e]
P_{Prot}, g/100 ml	4.1±0.34	4.0±0.31	−0.06 ±0.094[d]	−1.2 ±1.84%[d]
C_{Cr}, ml/min	67±4.9	67±4.8	−0.7 ±1.7[d]	0.0 ±8.3%[d]
C_{PAH}, ml/min	173±14	181±16	+6±13[d]	+4.6 ±7.2%[d]
Pa, mm Hg	100	102	+2—	+2%

Values are means ± SEM. Infusion = 2.7–4.0 μmoles/kg. Controls = values found over 20-min interval just before pronethalol infusion for 20–30 min into one renal artery (mean total dose of 3.2 μmoles/kg, with range of 2.7–4.0 μmoles/kg). After pronethalol = values found over first 20-min interval after stoppage of pronethalol infusion. Renal parameters refer to both kidneys. [a] $P < 0.001$. [b] $P < 0.01$. [c] $P < 0.05$. [d] $P > 0.5$, from paired t test. [e] $P > 0.1$. No. = 5 for all parameters, except Pa where $n = 1$. Dog weight: 16.4±3.1 kg. (mean ± SD).

ts was mean arterial blood pressure measured. As also shown in Table 5, the mean arterial pressure averaged 100 mm Hg in the 20-min control interval and 102 mm Hg in the 20-min interval of greatest inhibition of saline diuresis (0–20 min after stoppage of pronethalol administration). From this one experiment, therefore, it appeared that sizable reversals of saline diuresis can be evoked by pronethalol with little, if any, measurable reduction in mean arterial blood pressure.

Effect of infusions of nicotinate on lipokinetic and antinatriuretic effects of pronethalol. Noteworthy seemed the association of the pronethalol-induced reversal of the natriuresis of volume expansion with the drug-induced large elevations in plasma FFA levels. Previous work suggests that the intrinsic lipokinetic activity of pronethalol may be related intimately to the inhibitory effect of pronethalol on sodium diuresis, perhaps by furnishing more FFA as renal metabolic substrate or by stimulation of renal adenyl cyclase activity (38). Therefore, experiments were designed to attempt to dissociate the lipokinetic effect of pronethalol from its effect of reversal of saline diuresis.

Nicotinic acid was employed for this purpose, since nicotinic acid inhibits both pronethalol-induced and catecholamine-induced elevations in plasma FFA levels in dogs without evident cardiovascular adrenergic receptor-blocking activity (7, 16). This lipolytic inhibition is apparently medi-d by inhibition of induced adenyl cyclase activity and ition of formation of adenosine 3′,5′-monophosphate in adipose tissue (28, 29). However, nicotinic acid is optimally given parenterally in the neutralized form of sodium nicotinate, and large amounts of about 200 mg/kg (1.6 mmoles/kg) seem required for appreciable inhibition of pronethalol-induced lipokinesis in whole dogs (16, 38). Potentially complicating factors in studying saline diuresis during sizable administration of sodium nicotinate are: *1*) the direct vasodilatory effect of nicotinate (19); *2*) the extra amount of sodium ion given; and *3*) the major renal excretion of nicotinic acid as unmetabolized anion at high dose levels (19), with concomitant excretion of cation. Therefore, sodium nicotinate was given at a constant rate, because these experiments utilized an essentially steady state of sodium diuresis during sustained volume expansion as the control parameter. Sodium nicotinate solution was administered continuously by vein at 26.7 mg/min per dog (184 μmoles/min per dog), after injection of a priming dose of 75 mg per dog 40–70 min before the beginning of the control periods of saline diuresis. Infusion of inorganic isotonic saline solution was maintained at 0.4 ml/kg per min.

Three experiments were performed with the combined sodium diuresis of volume expansion and continuous nicotinate infusion. Mean arterial blood pressures and heart rates were measured in these experiments. The C_{PAH} was not an effective index of renal plasma flow in these experiments. This is because nicotinic acid in high doses depresses C_{PAH}, apparently by interfering with tubular PAH secretion, as previously observed (38). The C_{PAH} was measured in only one of the three experiments, and the C_{PAH} values exceeded C_{Cr} values only by about 30%.

The results are summarized in Table 6. In the control saline diuretic periods, renal sodium excretion averaged 813 ± 88 μEq/min, fractional sodium excretion averaged 9.8 ± 1.3%, urine flow averaged 4.26 ± 0.44 ml/min, and plasma FFA concentration was low at 0.17 ± 0.03 mM (mean ± SEM). Following the intravenous infusion of 4

TABLE 6. *Dissociation of antinatriuretic and lipokinetic effects of pronethalol during saline diuresis by continuous nicotinate infusion*

Mean Values	Controls Before	After Pronethalol	Paired Change	Paired Percentage Change
$U_{Na}V$, μEq/min	813 ±88	466 ±77	−343 ±19[a]	−43 ±3.2%[a]
F_{Na}Excr, %	9.8 ±1.3	6.0 ±1.1	−3.8 ±0.17[a]	−39 ±1.1%[e]
V, ml/min	4.26 ±0.44	2.23 ±0.58	−2.04 ±0.15[a]	−49 ±2.5%[a]
P_{FFA}, mM	0.17 ±0.02	0.40 ±0.15	+0.24 ±0.14[b]	+133 ±60%[d]
C_{Cr}, ml/min	58 ±1.5	55 ±1.2	−1 ±2.1[c]	−4.5 ±2.3%[d]
C_{PAH}, ml/min	78	68		
Pa, mm Hg	108±1.2	102±2.4	−6±2.1[d]	−5.8 ±1.7%[f]
HR, beats/min	108±11	117±4.1	+8±6.6[b]	+10 ±9.7%[b]

Values are means ± SEM. Controls = values found over 20-min interval immediately before pronethalol infusion intravenously for 20 min (4 μmoles/kg). After pronethalol = values found over first 20-min interval after stoppage of pronethalol infusion. Isosmotic sodium nicotinate was infused at 184 μmoles/min, after a priming dose of 515 μmoles/kg given 40–70 min before the control periods. Isosmotic saline infusion was also sustained, at 0.4 ml/kg per min. [a] $P < 0.01$. [b] $P > 0.2$. [c] $P > 0.5$, from paired t test. [d] $P > 0.1$. [e] $P < 0.001$. [f] $P > 0.05$. No. = 3 for all parameters, except C_{PAH} where no. = 1. Dog weight 15.0±1.3 kg (mean ± SD).

μmoles pronethalol per kilogram, sodium excretion was reduced by 43 ± 3.2% (mean ± SEM, $P < 0.01$), fractional sodium excretion was inhibited by 39 ± 1.1% ($P < 0.001$), and urine flow was reduced by 49 ± 2.5% ($P < 0.01$). On the other hand, the average rise of plasma FFA level of 0.24 ± 0.14 mM was not significantly different ($P > 0.2$) from the antecedent control mean FFA concentration in the paired experiments. With this pronethalol-induced reversal of the natriuresis, C_{Cr} did not measurably change, the mean change was −1 ± 2.1 ml/min ($P > 0.5$). Similarly, the slight average rise in heart rate of 8 ± 6.6 beats/min (mean ± SEM) was not statistically significant ($P > 0.2$). However, mean arterial blood pressures declined by 6 ± 2.1 mm Hg ($P > 0.1$) from values of 108 ± 1.2 mm Hg (mean ± SEM). The very slight arterial pressure reduction of 6 mm Hg on the average appeared doubtfully an important pressure or hemodynamic factor within the kidney in the kidney's large antinatriuretic response. Nevertheless, this seemingly slight average hypotensive effect of the 4 μmoles pronethalol/kg appeared as a possible clue, representing some significant action of pronethalol in its mediation of the suppression of natriuresis. Possibly noteworthy was the percentage reduction in mean arterial pressure following the pronethalol infusion, for it averaged 5.8 ± 1.7% (mean ± SEM). However, this percentage change in blood pressure was equivocally and not clearly of significance in these three experiments, for the P value was between 0.05 and 0.1.

Effect of butoxamine and N-isopropylmethoxamine on lipokinetic and antinatriuretic effects of pronethalol. It was also sought to inhibit the lipolytic action of pronethalol, given intravenously at 4 μmoles/kg, by use of two somewhat specific drugs which block the lipolytic and other metabolic actions of catecholamines. This was in order to attempt to dissociate the suppression of saline diuresis by pronethalol from the lipokinetic effect of pronethalol. The drugs used were *N-tert*-butylmethoxamine (butoxamine) and *N*-isopropylmethoxamine (IMA), in racemic form. Butoxamine is said to also antagonize vascular beta-adrenergic but not alpha-adrenergic responses (9, 27). *N*-isopropylmethoxamine is said to be without significant cardiovascular beta-adrenergic receptor blocking or stimulating activity (26).

Each agent was injected intravenously over 2.5 min in a dosage of 26 μmoles/kg (8.0 mg butoxamine/kg and 7.5 mg IMA/kg) in different dogs during sustained saline diuresis, about 45 min before institution of pronethalol infusion or 20 min. Following butoxamine pretreatment, pronethalol infusion still resulted in appreciable lipokinesis, for mean plasma FFA concentration rose by 0.7 mM, and some suppression of natriuresis also resulted. In the IMA experiment, 5 mg/kg of phenoxybenzamine (PBZ) were given intravenously just before injection of IMA. The PBZ prevented the hypertensive side effect of this dosage of IMA, which otherwise soon results, due to metabolic conversion of part of the IMA, to methoxamine, an agent which stimulates alpha-adrenergic receptors (6, 26). Pretreatment with PBZ-IMA prevented neither the lipolytic effect nor the antinatriuretic effect of pronethalol, 4 μmoles/kg. These results are illustrated serially in Fig. 2.

Larger doses of butoxamine and IMA were not investigated because of the potentially complicating possibilities of cardiodepression (27) and other limiting pharmacological effects (6). Probably relevant to the failure of butoxamine, 8 mg/kg, to inhibit appreciably the lipolytic effect of pronethalol is the much lesser inhibition by butoxamine of isoproterenol- and norepinephrine-induced lipolysis than of epinephrine-induced lipolysis (9).

Effect of phenoxybenzamine pretreatment on effects of pronethalol infusion during saline diuresis. In order to determine whether the administration of pronethalol, in dosage of 4 μmoles/kg, might cause significant hypotension if the baroreceptor-sympathetic nervous system control mechanism for arterial blood pressure homeostasis were inhibited, the alpha-adrenergic blocking agent, PBZ, was employed. In four experiments, PBZ was injected intravenously in dosage of 2 mg/kg 50–60 min before institution of intravenous infusion of pronethalol. In a fifth experiment, in which IMA was also given (vide supra), 5 mg PBZ/kg were administered. The results in the latter experiment are shown serially in Fig. 2, and the results in all five experiments are summarized in Table 7.

With the pretreatment with PBZ, the control arterial blood pressures during the sodium diuresis of volume expansion with saline were rather low and averaged 90 ± 7.5 mm Hg (mean ± SEM). However, renal sodium excretion averaged 425 ± 106 μEq/min, and fractional sodium excretion averaged 4.2 ± 0.98%. The infusion of pronethalol resulted in a significant reduction of arterial pressure, which changed by −13.6 ± 3.3 mm Hg (mean ± SEM, $P < 0.02$) and which changed percentagewise by −14.5 ± 2.5% ($P < 0.01$). The mean increase of heart rate of 9 ± 11 beats/min was not significant ($P > 0.2$). In association with the blood pressure change, renal sodium excretion was re-

TABLE 7. *Revelation by prior alpha-adrenergic blockade of combined antinatriuretic and hypotensive effects of pronethalol (4 μmoles/kg) during saline diuresis*

Mean Values	Controls Before	After Pronethalol	Paired Change	Percentage Change
$U_{Na}V$, μEq/min	425±106	241±76	−164±47[a]	−44±9.1%[a]
$F_{Na}Excr$, %	4.2±0.98	2.5±0.71	−1.7 ±0.67[b]	−44±12%[a]
V, ml/min	2.43 ±0.59	1.20 ±0.38	−1.21 ±0.36[a]	−51±8.1%[a]
P_{FFA}, mM	0.53 ±0.06	1.29 ±0.15	+0.76 ±0.096[c]	+148 ±19.5%[c]
P_{Prot}, g/100 ml	4.5 ±0.16	4.2±0.11	−0.26 ±0.027[c]	−5.9 ±0.62%[c]
C_{Cr}, ml/min	70 ±5.5	67±4.0	−3.5 ±2.6[d]	−4.3 ±2.3%[f]
C_{PAH}, ml/min	189±21	201±27	+12 ±13[d]	+5.1 ±6.6%[d]
Pa, mm Hg	90±7.5	76±5.0	−13.6 ±3.3[a]	−14.5 ±2.5%[a]
HR, beats/min	133±13	142±8	+9±11[d]	+9.4 ±9.1%[d]

Values are means ± SEM. Phenoxybenzamine (2 mg/kg in 4 exp and 5 mg/kg with 7.5 mg IMA/kg in 1 exp) was given 50–60 min before start of the pronethalol infusion. Controls = values found over 20-min interval just before pronethalol infusion intravenously for 20 min. After pronethalol = values found over first 20-min interval after stoppage of pronethalol infusion. [a] $P < 0.05$. [b] $P < 0.1$. [c] $P < 0.01$. [d] $P > 0.2$. [e] $P < 0.02$. [f] $P > 0.1$. Dog weight: 14.1 ± 1.6 kg (mean ± SD).

duced an average of 44 ± 9.1% ($P < 0.01$), and fractional sodium excretion was reversed by 44 ± 12% ($P < 0.05$). The average change in C_{Cr} of −3.5 ± 2.6 ml/min and average change in C_{PAH} of +12 ± 13 ml/min with pronethalol administration were not significant ($P > 0.2$). With the pronethalol-induced suppression of saline diuresis, plasma protein concentration fell slightly by 0.26 ± 0.027 g/100 ml (mean ± SEM), and plasma FFA level rose an average of 0.76 ± 0.096 mM.

DISCUSSION

In this study, it was demonstrated that neither the renal arterial infusion of palmitate in amounts which locally raised estimated plasma palmitate concentration by about 0.23 mM nor the renal arterial infusion of octanoate in amounts which locally raised plasma octanoate level as high as about 1.8 mM resulted in any local (or contralateral) suppression of the sodium diuresis of volume expansion with saline. In experiments with combined sodium diuresis of inorganic saline infusion and of continuous sodium nicotinate infusion, the administration of pronethalol markedly reversed the natriuresis, and this antinatriuretic effect of pronethalol was dissociated from its usually accompanying lipokinetic effect, as measured by arterial plasma FFA levels.

In experiments with renal arterial infusions of pronethalol, ranging from near threshold doses for natriuretic reversal to doses which caused suppression of saline diuresis by an average of about one-half, no local renal action of the pronethalol was demonstrable, for the inhibition of the sodium diuresis in the locally infused kidney was not significantly different from that observed simultaneously in the contralateral kidney. Even if pronethalol has a low renal plasma extraction ratio, it seems highly unlikely that the bilaterally observed antinatriuretic effects are predominately due to a local direct action of pronethalol on each kidney. This is because, with blood dilution and recirculation of pronethalol, one would expect that pronethalol would be delivered in the arterial blood to the contralateral kidney at a much lower concentration than that of the arterial blood of the kidney receiving the local arterial infusion of pronethalol. In addition, tissue distribution studies have shown that other tissues besides the kidney take up pronethalol to much higher concentration than that of the simultaneous blood concentration (4).

These above results collectively afford strong evidence against the hypotheses that *1)* plasma FFA as renal metabolic substrate antagonizes or inhibits intrarenally the natriuresis of volume expansion with saline (37, 38), and *2)* systemically administered pronethalol or other lipokinetic aromatic amine with mainly beta-adrenergic cardiovascular properties (40) inhibits renal sodium excretion mainly by a direct renal beta-adrenergic effect, either hemodynamically or through direct stimulation of tubular reabsorption of sodium (15, 17, 24, 25, 38).

The herein-described findings that unilateral renal arterial infusions of pronethalol in amounts up to 4 μmoles/kg/min resulted in marked reversal of the sodium diuresis of concurrent saline infusion to a similar degree in ipsilateral and contralateral kidneys, without significant change in GFR and effective renal plasma flow, are rather similar to previous findings with unilateral renal arterial infusions of isoproterenol and orciprenaline (metaproterenol) (21). The latter two agents are beta-adrenergic stimulating rather than blocking drugs (33). These findings, plus the previous observations that both dichloroisoproterenol and pronethalol in lipokinetic doses (but not the beta-adrenergic blocking agent propranolol) inhibited the development of saline diuresis (38), and the finding that the beta-adrenergic blocking agent Sotalol (MJ-1999) failed to reverse established saline diuresis (unpublished data) afford evidence that the antinatriuretic effect of pronethalol is intimately related to its intrinsic beta-adrenergic-like stimulating action. The dissociation of its lipolytic effect from its antinatriuretic effect by nicotinate treatment suggests also that the antinatriuretic effect of pronethalol may not be related to other intrinsic metabolic actions of this drug.

By tentative exclusion, the possibility then remains that the suppression of natriuresis by pronethalol is somehow related to one or more beta-adrenergic-like stimulating actions on the cardiovascular apparatus. Beta-adrenergic stimulation is well known to cause vasodilation and systemic hypotension, as is pronethalol (5, 35). Furthermore, even dose rates 2.5 times greater than the maximal dose rates of pronethalol (0.2 μmole/kg per min) given in these experiments do not usually decrease cardiac output in anesthetized dogs (35). These facts and the demonstration that an equivocal or statistically insignificant slight reduction in arterial pressure from antinatriuretic doses of pronethalol could be converted to a significantly measurable, mild, but greater reduction in arterial pressure by phenoxybenzamine pretreatment raise the possibility that the suppression of the natriuresis of volume expansion by pronethalol may be intrinsically related to its vasodilator effect. The possibility that this antinatriuretic response may be mediated by an arterial depressor effect with baroreceptor-induced reflex effects is not yet answered. Renal nerve activation may alter intrarenal hemodynamics and perhaps sodium excretion, even without change in total renal blood flow (30). Although the experiments with alpha-adrenergic receptor inhibition induced by PBZ, given intravenously, tend to mitigate the possibility that renal sympathetic nerve or neuroadrenomedullary activation may be involved in the antinatriuresis, such neural activation is not excluded because: *1)* in the PBZ experiments, the renal perfusion pressure was much lower following pronethalol infusion, and *2)* for essentially complete intrarenal adrenergic blockade, doses of PBZ as high as 15 mg need to be injected directly into the renal artery of dogs, and renal arterial injections of one-third this amount of PBZ only results in mild intrarenal adrenergic blockade (20). It is also possible that intravascular translocations of blood volume, more than simply a reduction of arterial pressure due to induced vasodilation, are involved in the pronethalol-induced suppression of the natriuresis of volume expansion. It is noteworthy that before mean arterial pressure is lowered, normally blood volume must change by hemorrhage by more than 10–20% (8).

The major factor or factors initiating the sodium diuresis of volume expansion with saline is still moot (13, 23). The inhibition and reversal of saline diuresis by pronethalol may conceivably be due to inhibition of release of a natriuretic hormone (11, 32). If this is the mechanism of the suppression

of natriuresis in these experiments, however, one must deduce that the natriuretic hormone has a very short biological half-life, for the antinatriuretic action of pronethalol developed very promptly after its administration.

Unfortunately, with use of butoxamine and IMA in dosage of 26 μmoles/kg, the plasma FFA elevations induced by pronethalol, 4 μmoles/kg, were not appreciably inhibited, and saline diuresis was suppressed by the pronethalol. These two metabolic blocking agents, therefore, did not appear very useful in attempting to dissociate the lipolytic and antinatriuretic effects of pronethalol during saline diuresis.

Relevant to the demonstration that renal arterial infusions of palmitate and octanoate did not augment tubular sodium reabsorption during volume expansion is the fact that renal uptake of FFA is usually linearly related to its arterial plasma concentration (2, 22). Thus, no direct support is found in these experiments for the hypothesis that restriction of uptake or oxidation of FFA as the source of energy for tubular sodium transport underlies regulation of renal sodium excretion (2, 38).

Barac-Nieto and Cohen (2) have suggested that the renal organic acid (e.g., PAH) transport system may be operative normally in the entry and/or utilization of endogenous plasma FFA by the kidney. No positive support for this hypothesis was found when endogenous FFA levels in plasma were elevated by pronethalol administration an average of about 0.8 mM (Table 5) or to values as high as 1.7 mM (Fig. 2), since neither mean renal C_{PAH} nor mean tubular C_{PAH} ($C_{PAH} - C_{Cr}$) was depressed concurrently. Depression of tubular clearance of PAH at the low plasma PAH levels (e.g., 1.4 mg/100 ml or about 0.07 mM) used for measuring effective renal plasma flow by renal C_{PAH} might be anticipated (by competition), if the renal uptake of FFA were on account of a similar or stronger affinity of FFA than PAH for the organic acid transport system and if the amount of plasma FFA extracted were great enough. On the other hand, all five major endogenous FFA (long) in dog plasma are poorly extracted by the kidney and of these, the most actively extracted one is palmitate (18). Its renal extraction is said to average only about 15%, and the arterial plasma level of palmitate normally averages about 0.1 mM in anesthetized dogs (18). Nevertheless, tubular C_{PAH} was not reduced in the renal arterial infusion experiments which increased locally plasma levels of palmitate by about 0.2 mM.

In contrast, octanoate, an intermediate carbon chain length FFA, is more readily extracted and oxidized by the dog kidney than the normal endogenous FFA usually measured by conventional techniques for plasma FFA analyses (18). These techniques (2, 38) predominantly measure long carbon chain length FFA in the plasma (12).

With exogenously induced local elevation in renal plasma octanoate level above about 0.4 mM, the tubular clearance of PAH at low plasma levels of PAH was depressed. The experimental dose-depressive response relationships strongly suggest that this inhibition of tubular C_{PAH} by octanoate represents an actual inhibition of tubular PAH secretion rather than a reduction in peritubular plasma flow of PAH. This is perhaps best illustrated in the form shown in Fig. 1. This figure relates the plasma molar concentration ratio of octanoate/PAH to the observed percentage inhibition of tubular C_{PAH}.

The curvilinear characteristic of this in vivo relationship shown in Fig. 1 is strikingly similar to the in vitro inhibition of PAH accumulation found in kidney slices as a function of the molar concentration ratio of octanoylglycine/PAH or hippurate/PAH in the medium (31). The somewhat lesser PAH inhibition found in our in vivo study at the same molar concentration ratio found in the in vitro study (31) is perhaps related both to the incomplete renal extraction of octanoate from flowing blood and to partial renal disappearance of octanoate due to oxidation (18), in place of maintenance as tissue octanoate (10) and/or in place of acyl conjugation to glycine (31). This in vivo similarity to the in vitro relationship (31) is presented as suggestive evidence that when an intermediate length FFA such as octanoate is delivered in the blood in adequate amounts to the tubular cells in vivo, intermediate length FFA does inhibit, and competitively so, tubular PAH secretion. The marked in vitro inhibition of renal PAH transport by octanoate and certain other intermediate carbon chain length ($C_7 - C_{10}$) FFA is well documented (10). However, lauric acid (C_{12}) and caproic acid (C_6) inhibit PAH accumulation in vitro poorly (10), and the authors do not know of any rather direct evidence (e.g., in vitro) that longer carbon chain length FFA, such as the major FFA in plasma (mostly C_{16} and C_{18} FFA) (18), inhibit tubular transport of PAH. The apparent depression of tubular secretion of PAH in our octanoate experiments in dogs may be directly by octanoate antagonism (10) and/or directly by renal conversion of octanoate to acylglycine as the antagonist of PAH for organic acid transport (31). Our results suggest that the organic acid transport system formulation for FFA by Barac-Nieto and Cohen (2) should perhaps be restricted to intermediate carbon chain length FFA such as octanoate.

It is interesting to note that *N*-methylnicotinamide, a principal metabolic and excretory product of nicotinic acid and nicotinamide in the dog (3), does not compete with PAH for tubular secretion (3). It seems, therefore, likely that the incidentally observed depression of renal C_{PAH} (and tubular C_{PAH}) by nicotinate infusion is by a similar type of competitive action of nicotinic acid on tubular secretion of PAH by the organic acid transport system.

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THE ACTIONS OF METABOLIC SUBSTRATES AND INHIBITORS ON THE RABBIT AURICLE*

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Knowledge of cardiac muscle metabolism has advanced during the past few years to an appreciable extent, but the correlation of metabolic processes with the various functions of the heart has been very little investigated. It is possible that some of the enzyme systems that are highly active in cardiac extracts or homogenates, or even in heart slices, are not intimately involved in the beating of the heart. It is also possible that in the intact beating heart there are enzyme systems of great importance, which are either destroyed or altered by any damage to the myocardium. Such systems may be brought to light by a more detailed analysis of the actions of metabolic substrates and inhibitors on the normally contracting cardiac tissue. For the understanding of drug actions on the heart, it is frequently necessary to correlate the metabolism with the functional aspects of impulse discharge, conduction, and contraction. The isolated rabbit auricle was used in the present work because both the amplitude of contraction and the rate of impulse discharge could be more accurately measured with it than with the entire heart where there occur problems of conduction block and complex contractile movement. It is also difficult to maintain normal conditions in the perfused heart, and the tissue soon becomes damaged, whereas auricles will beat regularly for many hours in the proper medium. This work was done in connexion with the problem of the action of acetylcholine on the heart and of how this action is influenced by the metabolic state of the myocardium. The results obtained with acetylcholine will be reported in a subsequent paper.

METHODS

The effects of metabolically important substances on the rabbit auricle were determined by addition of the substances to a bath in which the auricles were beating. Hearts from young rabbits were used, inasmuch as it was found that such preparations beat more regularly and give more reproducible results than those from older rabbits. It is possible that in older animals the auricular walls are so thick that diffusion of oxygen into the tissue, or diffusion of metabolic products from the tissue, is not adequate. It also seemed that better results were obtained when the rabbits were allowed to remain quiet for several minutes before they were killed. Auricles obtained from animals that had been killed immediately after being caught and handled often gave poor results

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Cap. Acid

and would seldom achieve a regular beat. The rabbits were killed by a blow on the head and the hearts were rapidly removed. The hearts were allowed to beat for about half a minute in a dish of physiological medium in order to allow as much blood as possible to be pumped out of the tissues. The ventricles were often massaged in order to rinse the blood from the auricles, care being taken not to touch the auricles. The auricles were then cut from the heart and carefully trimmed so that no ventricular tissue remained. Caution was exercised in the trimming of the arteries and veins that no damage be done to the adjacent regions of the auricle, for it is in these regions that the pacemaker tissue usually lies. The auricles were suspended in 70 ml. of Tyrode solution (0.137 M-NaCl, 0.0027 M-KCl, 0.0018 M-$CaCl_2$, 0.00011 M-$MgCl_2$, 0.012 M-$NaHCO_3$, 0.00042 M-NaH_2PO_4, and 0.0055 M-glucose) which, after saturation with the gas mixture, had a *p*H of 8.1. Substances to be tested were added in 0.7 ml. volumes at a concentration 100 times that required in the final medium. Although this medium is a good buffer, all solutions of these substances were brought to a *p*H close to 8.1 before their addition to the bath. All measurements were made at 30° C. It was found that better results could be obtained at this temperature than at 38° C. At the higher temperature it is likely that the rapid rate often limits the recording of the full amplitude and this would introduce serious errors into the determinations of amplitude effects. The auricles also beat regularly over a much longer period of time at the lower temperature. The medium was continually saturated with a mixture of 5 per cent CO_2 and 95 per cent O_2. The substances used were obtained in as pure a form as possible. The sodium fluoroacetate was kindly supplied by Dr. Saunders, of Cambridge University.

In order to achieve quantitative results the auricular tracings were carefully measured, the amplitude being recorded in millimetres and the rate of beating in contractions per ten seconds. It was necessary to use this small time unit for measuring the rates, inasmuch as rapid rate changes could not have been accurately determined if longer intervals had been chosen. The effects produced by the addition of any substance were expressed in terms of the percentage change in the amplitude and rate. Such results might conceivably be expressed as absolute changes, percentage changes, or as final levels of activity to which the auricles were brought by the substances. For most purposes the percentage change is the most significant. In the first place, owing to the recording arrangement, the true amplitude of contraction is not measured; hence, if absolute changes were given, it would not be possible to compare one experiment with another. Furthermore, in considering the effects of substances on the auricular frequency we are usually interested in the changes in the rates of processes occurring within the pacemaker cells. It is likely that the rates of such processes are roughly proportional to the frequencies of beating. In metabolic studies the effect of an inhibitor on an enzymatic system is usually expressed in terms of percentage inhibition, and so here we might expect a more direct relationship to the underlying processes if we also express the frequency changes in similar terms. Justification for this method of expression, with regard to both rate and amplitude, was found in the quantitatively comparable results obtained from auricles beating at greatly different rates and amplitudes.

The auricles usually beat somewhat irregularly for 5–30 minutes after placing them in the bath, but eventually became quite constant with respect to both rate and amplitude. Experimental work was always initiated after the auricles had reached this steady state. Such auricles would beat for two to three days, the amplitude gradually decreasing during the second and third days. No auricles were considered to be normal after the first day and all results reported were obtained on fresh auricles. The recorded amplitude was usually adjusted to between 40–60 mm. Normal auricles beat spontaneously at an average rate of 102 contractions per minute, although there was quite large variation between the auricles from different animals in this respect, the extremes recorded being 72 and 144. At 38° C. the frequency is approximately 80 per cent

greater than at 30° C., and thus the rate at body temperature would have been approximately 184 beats per minute, a figure not far distant from the normal *in situ* rate of the rabbit heart.

RESULTS

The effects of metabolic substrates on normal auricles

It is obvious that if a substance is to alter the beating of the auricles by acting through metabolic mechanisms, its quantitative effect will depend on the metabolic state of the myocardial cells. If a particular substrate or intermediate, which is of importance in the functional metabolism of the heart, is deficient, we might expect its addition to produce an effect. Whether this would be stimulation or depression cannot be predicted until the correlation between the various metabolic reactions and the functioning of the heart cells is better understood. On the other hand, if an enzyme system is saturated with respect to its substrate (that is, if the substrate is not limiting the rate of the reaction), there may be no response to the addition of the substrate, or there may perhaps occur a retardation of the enzyme systems that operate before the particular step in which the substrate is involved. Furthermore, the observed actions of substrates on the auricular tissue may be independent of enzymic mechanisms, the primary effect being upon the cellular membrane potential or the contractile substance itself. Lastly, the possible actions of these substances on the acetylcholine equilibrium in the cardiac tissue must be borne in mind. Since there is a slow formation of acetylcholine in the auricles (either within the heart cells themselves or released from the post-ganglionic nerve endings), it is possible that a substance may stimulate or depress this formation, or alter the rate at which this endogenous acetylcholine is destroyed by modifying the activity of the cholinesterase, or antagonize the action of the acetylcholine on the auricular cells.

The results obtained on the actions of substrates on normal auricles are summarized in Table I. Changes of less than 5 per cent are probably not significant unless derived from experiments upon several auricles. An inspection of this Table will show that stimulation of either rate or amplitude is very seldom seen with any of the substrates investigated. It is likely that the only really significant stimulation is that produced by citrate on the rate. These substances, in general, have no effect on the normal auricle until a certain concentration is reached and then they begin progressively to depress it. This is seen in the concentration-action curves in Fig. 1. On the whole, it is probable that the enzymatic systems involved in providing energy for the beating of the auricles are not limited by any of the substrates tested.

The amplitude is more readily depressed by these substances than the rate. On the average, for any specific concentration of substrate, the depression of the amplitude is two to three times as great as the depression of the rate. However, substrates vary in this respect and it will be seen that acetate at lower concentrations depresses the rate as strongly as it does the amplitude. The maximum effect, in most instances, is reached from one to two minutes after the addition of the substrate to the bath. There is then, with the majority of substrates used, a partial or complete spontaneous recovery from this depression. This was observed with pyruvate, lactate, fumarate, malate, acetate, oxalacetate, propionate, and butyrate; it was not seen with citrate or caprylate, the depression produced by these being

TABLE I

THE EFFECTS OF METABOLIC SUBSTRATES ON NORMAL AURICLES

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Succinate	0.0001	1	0	0	1	1
		5	0	+2	1	1
	0.001	1	0	0	1	1
		5	0	+3	1	1
	0.005	0.5	0	−7	1	1
		1	0	−9	2	5
		2	0	−12	2	4
	0.01	0.5	−18	−22	3	3
		1	−13	−26	4	4
		2	−10	−27	3	3
Fumarate	0.001	0.5	−6	−4	1	1
		1	−6	−5	1	1
		2	−3	0	1	1
	0.005	0.5	−3	−5	2	2
		1	−3	−7	3	3
		2	0	−6	2	2
	0.01	0.5	−9	−8	2	3
		1	−5	−14	3	4
		2	−2	−10	3	3
Malate	0.001	0.5	−3	0	2	2
		1	−3	0	2	2
		2	−6	−1	1	1
	0.005	0.5	−2	−10	3	6
		1	−4	−14		5
		2	0	−15	3	3
	0.01	0.5	−4	−9	2	3
		1	−4	−18	2	3
		2	−4	−20	2	2
		5	0	−15	2	2
		10	0	−8	2	2
		15	0	−5	1	1
	0.02	0.5	−7	−23	1	1
		1	−14	−32	1	1
		2	−7	−27	1	1
		5	0	−22	1	1
		8	0	−12	1	1
		15	0	−8	1	1
Oxalacetate ..	0.002	0.5	0	−2	1	1
		1	0	−2	2	2
		2	0	0	1	1
	0.01	0.5	0	−9	1	1
		1	0	−14	1	1
		2	0	−13	1	1
		3	0	−11	1	1

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Pyruvate	0.0001	1	0	0	2	2
		2	0	−2	2	2
	0.001	0.5	0	−2	2	2
		1	0	−5	2	2
		2	0	−6	2	2
		5	0	0	2	2
		10	0	+3	2	2
	0.005	0.5	−1	−3	5	10
		1	−1	−6	7	13
		2	−1	−11	5	10
		3	−2	−9	3	5
		5	−2	−8	4	6
		10	−4	−5	3	3
		15	0	−3	2	2
	0.01	0.5	0	−9	2	2
		1	0	−12	4	4
		2	0	−16	5	5
		3	0	−7	2	2
		15	+5	−6	1	1
	0.02	0.5	0	−8	1	1
		1	−8	−3	1	1
		2	0	−23	1	1
Acetate	0.00002	1	0	0	1	1
		5	0	0	1	1
	0.0001	1	−6	−8	1	1
		2	0	−4	1	1
	0.0005	0.5	−9	−4	2	3
		1	−22	−15	2	3
		2	−17	−10	2	3
	0.001	0.5	−5	−9	4	5
		1	−19	−21	3	4
		2	−11	−9	3	3
		5	−3	−2	2	3
	0.01	0.5	−4	−8	2	2
		1	−6	−23	3	4
		2	−4	−16	2	3
		5	0	−7	2	3
		10	+6	−3	2	2
		15	+6	−2	2	2
	0.03	1	−6	−27	2	2
		2	−4	−22	2	2
		5	0	−18	1	1
		10	+6	−11	1	1
		15	+6	−15	1	1

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Propionate ..	0.0005	0.5	0	−1	2	2
		1	0	−4	2	2
		2	0	−3	2	2
		5	+3	−8	2	4
		10	+7	−3	2	3
	0.005	0.5	−7	−10	1	2
		1	−5	−7	1	2
		2	0	−3	1	2
		5	+5	−1	1	2
	0.01	0.5	−7	−4	1	1
		1	−7	−6	1	1
		3	0	−3	1	1
Butyrate	0.0005	1	0	−1	1	1
		5	0	0	1	1
	0.001	1	0	−10	1	2
		2	−3	−8	1	2
	0.005	1	0	−16	1	2
		2	−6	−11	1	2
		5	−3	−8	1	2
	0.01	0.5	0	−7	1	1
		2	0	−6	1	1
β-Hydroxy-butyrate	0.001	0.5	0	−4	1	1
		1	0	−6	1	1
		3	0	−10	1	1
	0.005	1	−7	−11	1	1
		3	0	−13	1	1
Caprylate	0.00002	0.5	0	0	1	2
		1	0	0	1	2
		3	0	−4	1	2
		5	0	−9	1	1
	0.0001	0.5	0	0	1	1
		1	0	−5	1	1
		3	−6	−16	1	1
		5	−6	−26	1	1
	0.0005	0.5	−6	0	1	1
		1	−17	−11	1	1
		5	−6	−24	1	1
	0.001	1	−7	−15	1	1
		2	−14	−25	1	1
	0.01	1	−22	−33	1	1
		2	−22	−42	1	1

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Glutamate ..	0.001	1	0	0	1	1
		2	0	0	1	1
	0.005	1	0	0	1	1
		2	0	0	1	1
	0.01	1	0	−3	2	3
		2	0	−1	2	3
	0.03	1	0	−7	1	1
		2	0	−5	1	1
Lactate	0.001	1	0	−2	1	1
		2	0	−1	1	1
	0.005	1	0	−1	2	2
		2	0	−1	2	2
	0.01	1	0	−6	2	2
		2	0	−2	2	2
		5	0	0	2	2
	0.03	1	0	−9	1	1
		2	0	−3	1	1
		5	0	−2	1	1
Citrate	0.0001	1	0	0	1	1
		2	0	0	1	1
	0.001	0.5	0	−5	2	3
		1	−2	−11	2	3
		2	−2	−12	2	3
	0.005	0.5	0	−28	2	3
		1	+3	−47	2	3
		2	+6	−60	2	3
		4	+2	−62	2	3
	0.01	0.5	0	−35	2	2
		1	+16	−63	2	2
		2	+25	−74	2	2
		5	+28	−75	2	2
		10	+25	−74	2	2
		15	+20	−72	2	2

irreversible until the substance was washed from the bath. Typical curves illustrating this spontaneous recovery are given in Fig. 2. This recovery is not due to destruction of the substrate, for in most instances the total amount of substrate present in the bath is sufficient to prevent any appreciable decrease in the concentration resulting from amounts destroyed by the tissue in the short time intervals involved. This type of spontaneous recovery is seen in cardiac tissue under the influence of a variety of substances, and is probably to be interpreted in the light of the ability of the cardiac functional systems to adjust themselves to abnormal conditions.

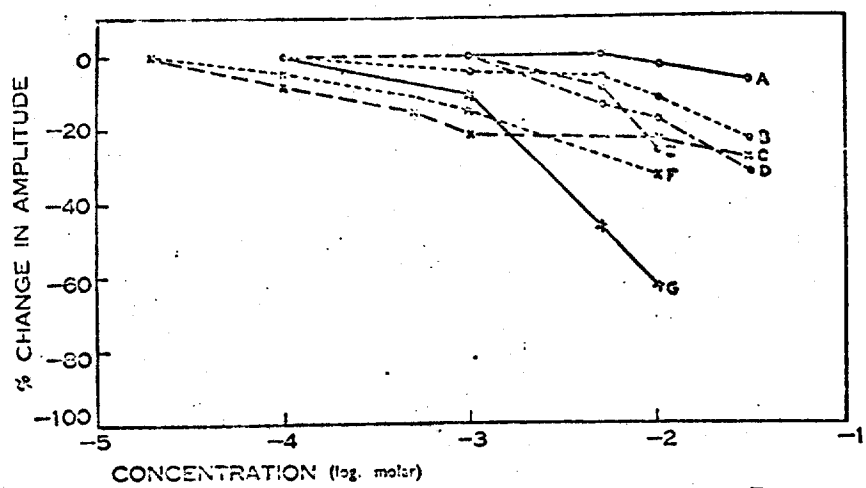

FIG. 1.—The action of substrates on the normal rabbit auricle. (A) glutamate, (B) pyruvate, (C) acetate, (D) malate, (E) succinate, (F) caprylate, (G) citrate. Time of action: 1 min.

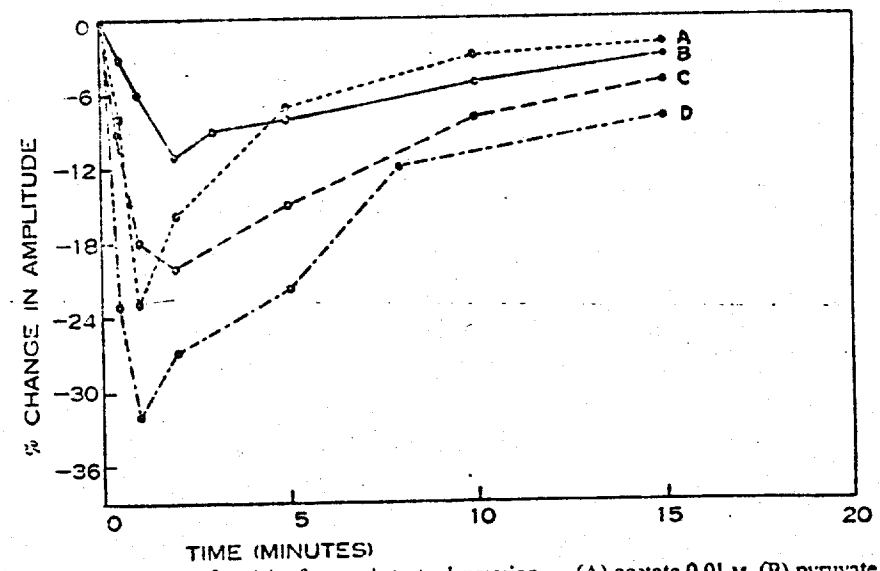

FIG. 2.—Recovery of auricles from substrate depression. (A) acetate 0.01 M, (B) pyruvate 0.005 M, (C) malate 0.01 M, (D) malate 0.02 M.

Consideration of the penetration of these substances into the cardiac cells is of the greatest importance, for, unless the heart cells, like some bacteria, possess enzymatic systems upon their surfaces, it is necessary for the substances to enter the cells in order to produce a direct effect upon the metabolism. Very little work has been done on the permeability of cell membranes to such substances. Further-

more, no analogies between the penetration of these substances and other weak acids can be drawn with confidence, inasmuch as cell membranes seem frequently to possess selective permeabilities for substances of functional importance. At the slightly alkaline *pH* of this medium, these substrates exist in solution preponderantly as anions: this may interfere with rapid penetration, especially when the substrates possess two or three such ionized groups. However, it must be borne in mind that the permeability to charged molecules may markedly increase in the heart cells during the passage of an impulse over the tissue. There is some evidence for the penetration of such substrates. It will be shown later that in substrate-depleted auricles there is an almost immediate effect of added pyruvate. Furthermore, the actions of pyruvate, lactate, β-hydroxybutyrate and other substrates are quite rapid on exhausted perfused hearts. It will also be shown that iodoacetate and fluoroacetate penetrate the auricular cells quite readily. With regard to dicarboxylic and tricarboxylic acids, I know of no evidence that is applicable to the present situation, although it seems certain that succinate and malonate are able to penetrate into heart slices. Fig. 1 shows no correlation between effect and the number of carboxyl groups. I believe that the evidence available points to a definite penetration by these substances. However, equilibrium may not be reached for one to three minutes, the times of maximum substrate action thus being expressive of the relatively slow rate of penetration compared to uncharged molecules.

It is possible that the effects observed with the substrates may have been partly or wholly non-metabolic effects. The relationship between cellular membrane potentials and the rate of discharge of the pacemaker cells or the degree of contraction is not understood, but it is conceivable that alterations brought about in the cell membrane by these substrates might influence the auricular functioning. Such organic acids are known to be capable of producing temporary electrical potential changes in nerve, muscle and plant cells. If such a mechanism of action is involved, it is likely that we must interpret the spontaneous recoveries from substrate depression as originating in the gradual restoration of the normal potential by the establishing of a concentration equilibrium between the medium and the interior of the cells. That these substrates depress the auricles by increasing in some manner the effective acetylcholine concentration in the tissue is unlikely, since it was clearly shown that atropine, at a concentration capable of blocking all acetylcholine action (1×10^{-5}), failed to alter the actions of pyruvate, citrate, acetate, malate, and fumarate. It is improbable that the depressant action originates in a simple increase in osmotic pressure of the medium, for glucose may be added up to a concentration of 0.03 M without alteration of the beat. Furthermore, if an osmotic effect were involved, we should expect the various substrates to produce similar degrees of depression at equivalent concentrations: this, however, is not the case. Since the concentration-action curves for the various substrates, as shown in Fig. 1, are not only at different positions along the concentration axis, but also possess markedly different curvatures, it would seem unlikely that the depressant effects were due to any general, non-specific type of action.

Some of the substrates, particularly acetate, malate, and propionate, showed a tendency to induce arrhythmias in the auricles. These arrhythmias were occasionally completely irregular, but usually they were of the "pulsus alternans" type, the amplitude of contraction regularly alternating each beat between two different

values. Sometimes such a rhythm was caused by the regular beating of one auricle of a pair while the other auricle beat only every other contraction, but more frequently this was not so. Rapid recoveries from such arrhythmias were observed when the substrate was removed from the bath.

The action of succinate was only depressant in these experiments. Depression of the amplitude was observed by Salant, Livingston, and Connet (1917) on the perfused frog heart (succinate 0.01–0.02 M) and by Forssmann and Lindstén (1946) on the isolated and perfused rabbit heart (succinate 0.01–0.04 M). They did not find in these preparations such marked depression of the rate as was here observed on the rabbit auricles. Forssmann and Lindstén (1946) found a mild stimulation (+15 per cent) of the amplitude by succinate in lower concentrations (0.0005–0.005 M). In the auricles no such stimulation was observed within a similar concentration range; this possibly indicates a difference in behaviour between the auricles and ventricles of the rabbit, inasmuch as they measured the contractions of the left ventricle. No stimulation was found in the isolated and perfused rat heart (Nakamura *et al.*, 1949). Despite the rapid oxidation of succinate by cardiac tissue, it would appear that the energy derived from this is, at least in the normal auricle, of no consequence in the functioning of the auricles. It will be shown later that this energy cannot be utilized to revive substrate-depleted auricles.

The relatively marked depression produced by malate on the auricles is in accord with the results of Salant, Livingston, and Connet (1917) on the frog heart; the frog heart would seem to be more sensitive to the action of this substrate than the rabbit auricle. A definite difference in the behaviour of the two tissues is seen, however, in the recovery of the rabbit auricles from the malate depression, whereas the frog heart was reported to remain in a constant state of depression as long as the malate was present (in one case for 62 minutes).

The action of citrate on the rabbit auricle is very interesting, there being a marked stimulation of the rate while the amplitude is simultaneously reduced. This phenomenon occurs only at higher concentrations of citrate (above 0.005 M); below this there is little effect on the rate, although the amplitude is depressed. The acceleration of the rate occurs quite suddenly. For example, in one experiment using 0.01 M-citrate, the rate was unchanged 20 seconds after the addition of the citrate, but it suddenly increased by 22 per cent at 40 seconds, and was up by 39 per cent at 60 seconds, after which it remained relatively constant. The depression of the amplitude observed here is very similar to that reported by Forssmann and Lindstén (1946) on the entire heart, but, unfortunately, they did not include their results on the rate changes. How much of the action of citrate was due to a decrease in the concentration of ionized calcium in solution is difficult to say. Added calcium will counteract the depression produced by citrate, but this does not prove that citrate acts by calcium depletion. When the concentration of citrate is 0.001 M or above, there must be a decrease in the ionized calcium, the original concentration of the calcium ion in the medium being 0.0018 M. If one considers the effect on the rabbit auricle of decreasing the calcium concentration (Webb, 1950), and compares this with the concentration-action curve for citrate depression of the amplitude, one must admit that the results coincide with what one might expect if citrate were acting by calcium depletion. On the other hand, the stimulation of the rate cannot be due to a decrease of ionized calcium in the medium, for low-calcium concentra-

tions have very little, if any, effect on the rate of the rabbit auricle. There still remains the possibility that the citrate penetrated the pacemaker cells and within them produced a disturbance in the calcium equilibrium, thereby producing changes in discharge rate that would not have occurred as the result of a mere decrease in external calcium.

The effect of glucose deficiency on rabbit auricles

If rabbit auricles were left for 24–48 hours at 30° C. in a glucose-containing medium a marked reduction in the amplitude was usually observed, especially if the medium had been continually replaced, although the rate often remained the same as it was originally. Under such circumstances the auricles stop in the relaxed condition of diastole. These auricles could usually be restarted with acetylcholine or adrenaline (Vane, 1948; Burn and Vane, 1949) but not by any of the substrates that were tried (pyruvate, acetate, succinate, oxalacetate, and malate). This type of exhaustion occurs in the presence of glucose and is probably not related in any way to depletion of substrate. It is likely that some alterations of the cellular enzyme systems have taken place, such as the depression of the formation of acetylcholine (Dullering and Burn, 1949) or a dissociation of the reactions of oxidation and phosphorylation.

If glucose was removed from the medium in which the auricles were beating, there was no immediate effect. It is to be expected that the auricles would have sufficient endogenous substrate—glycogen and fat—on which to operate for a period of time. The amount of work performed by beating auricles under these conditions was not great, for they were not working against the usual internal pressure of the blood. Seven experiments were performed in which the behaviour of the auricles in the absence of glucose was observed for five to seven hours. In three of these experiments there was no change of rate, and in four there was some decrease (-10 to -30 per cent). In three of these experiments there was no change in amplitude, and in four there was some decrease (-30 to -50 per cent). The auricles thus show variation in their behaviour in the absence of glucose, some apparently having a greater supply of endogenous substrate than others. Since there was essentially no change in rate or amplitude over a period of five to seven hours in the presence of glucose, these decreases in rate and amplitude, when they occurred, must be attributed to the lack of glucose. In longer experiments, when the auricles were left without glucose overnight, the exhaustion produced by substrate depletion is complicated by the simultaneous development of the type of exhaustion seen when glucose is present. Furthermore, it seems evident that lack of glucose in the medium produced some irreversible change or damage in the cardiac cells, for although stimulation was observed when glucose was added to such auricles, there was never anything like complete recovery. This type of damage may be related to that occurring when cardiac tissue is subjected to oxygen deficiency (Webb, Saunders, and Thienes, 1949a).

In order to produce rapid substrate depletion, with as little complication from other changes as possible, the auricles were driven with adrenaline (1×10^{-6}) in several experiments for varying periods of time in the absence of glucose. Such a procedure exhausted the auricles to a low level of activity in 15 to 60 minutes. If adrenaline were allowed to act for one to three hours, the auricles, after the

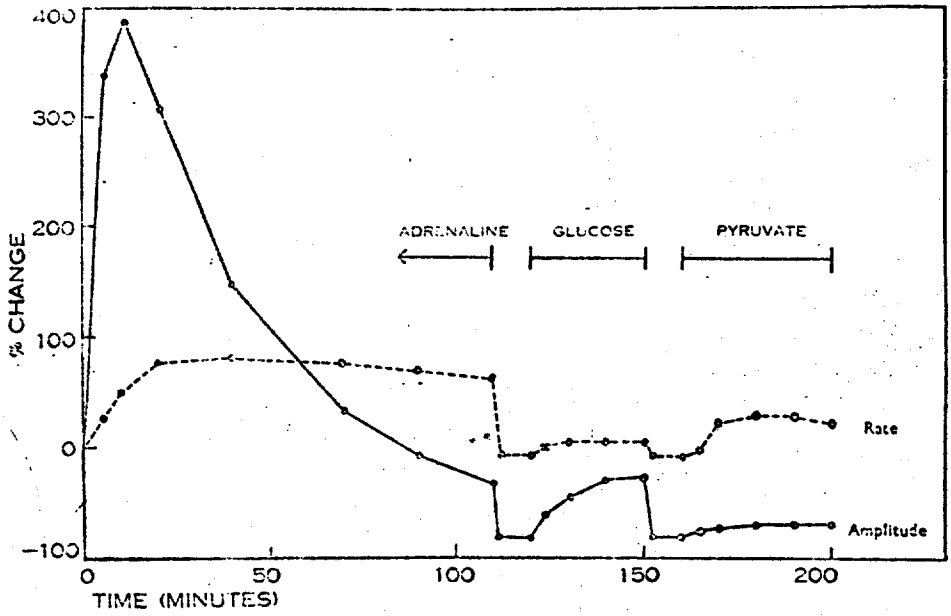

FIG. 3.—Substrate depletion of the auricles and the actions of glucose and pyruvate. Average of four experiments. Adrenaline, 10^{-6}; glucose, 0.0055 M; pyruvate, 0.005 M.

adrenaline had been washed out, beat with a very small amplitude, although the rate was usually relatively unaffected. The averaged results of four experiments are shown in Fig. 3. The rate in the presence of adrenaline remained at a high level, while the amplitude rapidly decreased to below the normal level. After the adrenaline had been removed, the rate returned to nearly its original value and the amplitude was very depressed. Exhaustion with adrenaline in the absence of glucose seems to affect the amplitude much more than the rate, the latter being quite stable. In spontaneous exhaustion in the absence of glucose the amplitude was also more easily depressed. The question whether adrenaline produces cardiac failure by depletion of substrate under such circumstances has never been settled. Some investigators have found that adrenaline-driven hearts lose their glycogen and attribute the failure to this, while others have shown that the heart may fail when there is sufficient glycogen present (Chang, 1936; Fieschi, 1933; Bogue, Evans, and Gregory, 1937; and others). This problem might be solved by determining the extent to which the cardiac tissue will recover when glucose is given. If there is complete, or nearly complete, recovery the failure may be attributed to substrate depletion, while if no recovery is seen it is likely that damage and disorganization have occurred to the functional systems. Any intermediate situation would indicate the extent of substrate depletion or cardiac damage. In the present investigation we shall see that the situation is intermediate and we must conclude that changes have taken place in the auricular tissue in addition to substrate depletion. However, for convenience we shall speak of such auricles as depleted.

When the auricles were driven with adrenaline there was invariably a gradual development of contracture as the amplitude decreased. This may also be expressed as an increase in tone or a decreased ability of the auricular muscle to relax. Auricles brought to almost complete exhaustion with adrenaline in the absence of glucose usually beat at a normal rate with a greatly diminished amplitude and were in a state of contracture. This inability to relax was also seen in the longer experiments when glucose was absent and no adrenaline was used, but it was never observed in exhaustion occurring when glucose was present. The problem of relaxation will be discussed after the results obtained with various metabolic inhibitors have been presented.

Effects of metabolic substrates on substrate depleted auricles

Auricles that had been spontaneously depleted or driven with adrenaline in the absence of glucose were invariably stimulated by the addition of 0.0055 M glucose, the concentration normally present in Tyrode solution. Furthermore, the degree of contracture of the auricles was lessened: that is, the auricles showed an increased ability to relax. Sometimes the degree of relaxation could be alternately increased and decreased by additions and removals of glucose. Complete recovery to the initial amplitude was never seen, but the rate, if it was decreased, was frequently restored to its normal value. The rate, however, in the depleted auricles was never far below its original value, while the amplitude was markedly depressed to only 10–20 per cent of the initial height. Usually there was a fairly rapid rise in amplitude over the first two minutes after glucose addition, and this was followed by a more gradual increase to reach a maximum value after 10–40 minutes. In terms of percentage, glucose increased the amplitude by 100–300 per cent over the depressed value. This is illustrated in Fig. 3, where the action of glucose may be compared with that of pyruvate.

Pyruvate likewise stimulated the substrate depleted auricles but not so well as glucose. However, the effect upon the rate was more marked with pyruvate, the rate being occasionally increased over the normal original value, while a good effect was sometimes obtained on the rate with little or no stimulation of the amplitude. The stimulation of the amplitude by pyruvate was usually between 10–60 per cent, and when compared with glucose on the same auricles this amounted to about one-fifth of the glucose effect. Pyruvate also induced relaxation of the auricles but was not so effective as glucose in this respect. The optimal concentration of pyruvate for such stimulation was found to lie between 0.001–0.005 M.

Only acetate and β-hydroxybutyrate of all other substrates tested were capable of producing any recovery in the substrate depleted auricles, and these effects were small and variable. Neither of these substances was found to induce relaxation. With acetate, the concentration had to be low (0.001 M) for any stimulation to be observed, for if higher concentrations were used only further depression resulted. In six experiments with 0.001 M-acetate, the average increase in amplitude was 13 per cent whereas the rate was not stimulated. Substrates with no ability to revive depleted auricles were fumarate, succinate, citrate, malate, oxalacetate, propionate, butyrate, and glutamate.

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It is interesting to note that glucose and pyruvate would occasionally facilitate relaxation of the auricular muscle although the auricles were not beating. Here we may confidently say that depletion of substrate increases the tone of the cardiac muscle and that the presence of glucose or pyruvate decreases the tone. When the auricles were beating, the amplitude of contraction usually decreased while the tone was increasing, and vice versa, but this was not always true, and we must conclude that there is no necessary relationship between the degree of contraction, and the ability of the muscle to relax. Failure of the muscle to relax sufficiently naturally inhibited the development of a full contraction, but it was observed occasionally, when glucose or pyruvate were added while the contracture was proceeding, that the amplitude may increase without simultaneous relaxation.

The results presented here on the rabbit auricle agree well with those obtained in the heart-lung preparation and in the isolated and perfused mammalian heart, not only with respect to the substrates that are utilizable by the cardiac tissue, but also with the comparative abilities of these substrates to revive the depleted heart muscle. However, on no other tissue are the differential actions of glucose and pyruvate on the rate and amplitude so easily observed. On may conclude that auricular and ventricular functional metabolism is qualitatively similar. It is likely, however, that somewhat different systems are involved in the discharge of impulses by the pacemaker cells and in the processes of contraction.

Effects of metabolic inhibitors on normal auricles

Curves showing the relationship between the concentration of inhibitor and the action produced are presented in Figs. 4 and 5. The time course of inhibitor

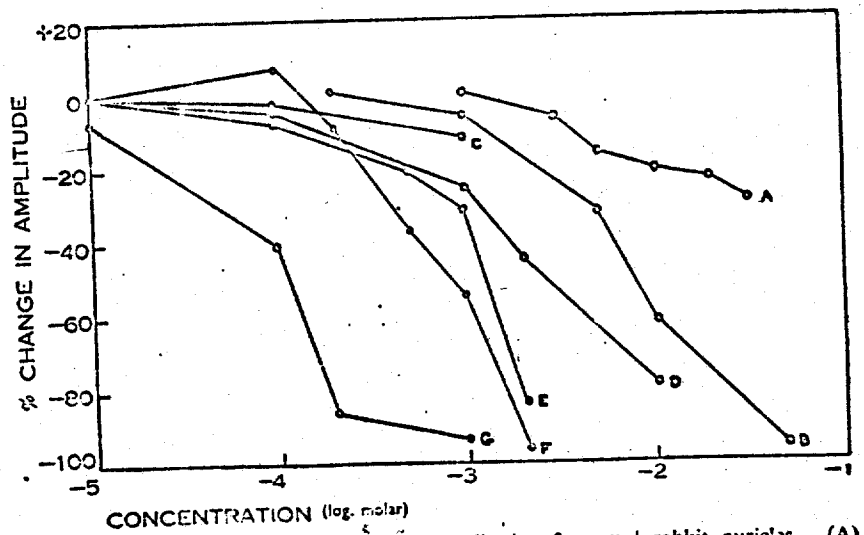

FIG. 4.—The action of inhibitors on the amplitude of normal rabbit auricles. (A) malonate—2 min., (B) fluoride—4 min., (C) phlorizin—5 min., (D) fluoroacetate—25 min., (E) azide—4 min., (F) cyanide—2 min., (G) iodoacetate—25 min. The times refer to the duration of inhibitor action.

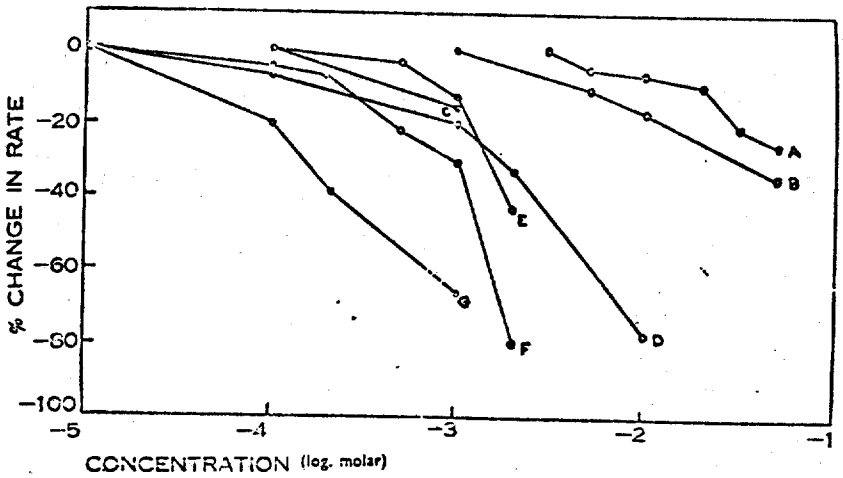

FIG. 5.—The action of inhibitors on the rate of normal rabbit auricles. Same designations as in Fig. 4.

action is illustrated in Figs. 6 to 12, where the actions upon rate and amplitude may be compared. These are all average curves obtained from three to six experiments.

Cyanide

Cyanide invariably produced a temporary stimulation before it began to depress the auricles, this stimulation being more marked in the amplitude than in the rate. The degree and duration of stimulation varied with the concentration of cyanide: at low concentrations (0.00001–0.0001 M) the stimulation was small but persisted, often not being followed by a depression, but as the concentration was increased the period of stimulation became shorter and shorter, so that at concentrations around 0.001 M the maximum stimulation was reached 20–40 seconds after addition and was followed immediately by a rapidly developing depression. Such a temporary stimulation was also seen when the auricles were made anoxic.

The rapid development of the depression would indicate that the available supply of high-energy phosphate bonds in the auricle, at least with respect to some process, is not great, unless cyanide, through some unrecognized action, prevented the direct utilization of this energy. A concentration of cyanide of 0.001 M produces a final depression of about 90 per cent in the amplitude and 50 per cent in the rate; this concentration was reported to inhibit the respiration of minced rabbit heart 45 per cent (Banga, Schneider, and Szent-Györgyi, 1931) and the respiration of rat heart slices 74 per cent (Webb, Saunders, and Thienes, 1949b). The rabbit auricle will usually stop in a concentration of 0.002 M-cyanide. It is probable that, when the auricles stop, there is still a definite fraction of the respiration proceeding; the level of activity to which the auricles are brought by any concentration of cyanide is not a quantitative measure of the cyanide-resistant respiration. Impulse discharge from the pacemaker cells was not inhibited so readily as the amplitude of contraction; this has been observed with other cardiac

preparations (Loewi, 1897; Beresin, 1913; and others). Most workers have reported that hearts poisoned by cyanide stop in diastole (Loewi, 1897; and others) but some have observed systolic standstill (Fujimaki, 1924). In the rabbit auricle both may be observed according to the conditions of the inhibition. When the auricles were stopped rapidly by higher concentrations of cyanide, they stopped in the relaxed condition of diastole; however, if the action of cyanide at concentrations just below those required to stop the auricles was prolonged, the auricles began to go into contracture and eventually stopped in systole. Occasionally both phenomena were observed in the same experiment. In one instance, the auricles were stopped in 80 seconds by 0.002 M-cyanide, but they slowly and partially recovered in the presence of cyanide, so that at 30 minutes the rate was depressed to only 40 per cent of its original value and the amplitude 92 per cent of its original value. At this stage the auricles were in a state of partial contracture, an inability to relax having been developed during this time. Eventually they stopped in systole. However, contracture is less commonly observed with cyanide than with inhibitors like iodoacetate, fluoroacetate, and fluoride.

Spontaneous recovery from depression in the presence of cyanide was very seldom observed, the example cited above being the only definite instance. Normally the auricles would reach a certain level of depression and remain there, or, if the depression were sufficient, they would slowly pass into a state of contracture. The auricular tissue seems to have very little ability to adjust itself to conditions in which the oxygen utilization is impaired: no ability to adjust to anoxic conditions was observed. During very slight cyanide depressions, there was occasionally some recovery, but this may be explained by loss of cyanide from the medium, some being carried off by the vigorous gassing. When the cyanide was removed from the medium, the auricles recovered completely, the rapidity of recovery depending on the concentration of cyanide and the duration of exposure. After marked depression following exposures of three to six minutes to 0.001 M-cyanide, the auricles would recover completely within ten minutes, but if the exposure time was only one minute the recovery took place within three to five minutes. No irreversible damage to the cells was observed at these concentrations, unless full contracture was allowed to occur. After the cyanide had been washed out a stimulation of the rate during the recovery phase was frequently observed, amounting to a 20–30 per cent increase over the normal rate before the cyanide had been added. Various arrhythmias commonly occurred during this recovery phase. It is possible that during the depression some energy-containing or energy-supplying substance had accumulated in the cells and that when oxygen was again available this resulted in a temporary stimulation of activity; this stimulation, however, was observed only with regard to the rate.

When excess calcium was added to the medium there was a marked stimulation of the amplitude, the rate remaining unchanged (Webb, 1950). Cyanide (0.001 M) antagonized this action, so that lower concentrations of calcium (0.0001–0.0005 M) were almost without effect, while the action of higher concentrations was markedly diminished. Cyanide also diminished the stimulating action of adrenaline on the auricles. In one experiment, adrenaline (10^{-6}) normally increased the rate by 69 per cent and the amplitude by 390 per cent, but in the presence of cyanide (0.001 M) the observed effects of adrenaline were zero and 105 per cent respectively.

Azide

It is interesting to compare the actions of cyanide and azide because of the usually accepted similarity of their enzymatic inhibitions. Azide was not as potent an inhibitor of the auricles as cyanide. In equal concentrations, azide acted more slowly than cyanide and at any particular time produced only 1/2 to 1/3 the depression that would have been produced by cyanide. In Figs. 6 and 7 comparison may be made of their actions at 0.0005 M. Azide did not produce the temporary stimulation observed with cyanide, nor did low concentrations ever stimulate. As with cyanide, azide depressed the amplitude more than the rate. This was also observed on the isolated hearts of cats and rabbits by Graham (1949).

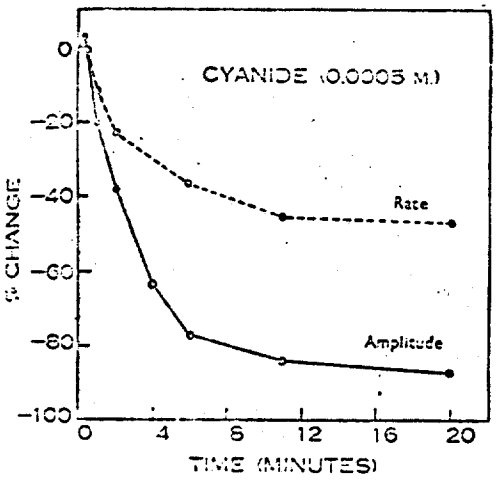

FIG. 6.—The action of cyanide on the rabbit auricle.

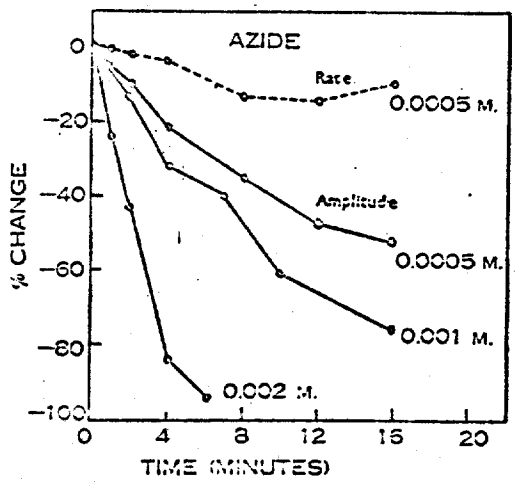

FIG. 7.—The action of azide on the rabbit auricle.

Recovery of the auricles after the azide had been removed was slower than with cyanide; 10 minutes were required after an exposure of 12 minutes to a concentration of 0.0005 M, and 20 minutes after a four-minute exposure to a concentration of 0.002 M, indicating that the azide is more tightly bound than cyanide.

Armstrong and Fisher (1940) concluded that cyanide and azide acted differently on fish hearts because the cyanide-stable respiration was different from the azide-stable respiration. However, this may be merely an expression of the wider range of action of cyanide at higher concentrations, for it is known that cyanide above a concentration of 0.001 M begins to inhibit dehydrogenases in addition to its action on metal-containing enzymes. However, it is clear that even in lower concentrations there was some difference in behaviour between the two inhibitors in their actions on the rabbit auricle.

Fluoride

Fluoride at concentrations between 0.005–0.01 M first depressed both the rate and amplitude of the auricles, but after approximately five minutes the amplitude began to increase and eventually reached values above the normal original level

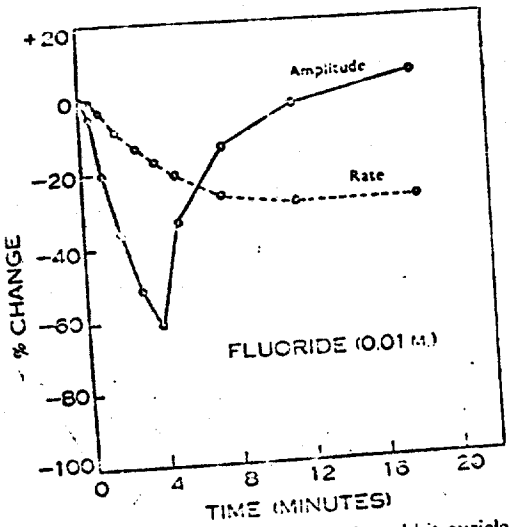

FIG. 8.—The action of fluoride on the rabbit auricle.

(see Fig. 8). Meanwhile, the rate remained at its depressed level or continued to decrease slightly. After 20–40 minutes the auricles were beating slowly but with a large amplitude. However, occasionally contracture began and the amplitude again decreased owing to the increasing inability of the auricles to relax. Previous reports on the action of fluoride on cardiac tissue have been equivocal. Several investigators have found only depression: Vernon (1910a, b) with 0.24–1.2 M-fluoride on the perfused tortoise heart, De Nitto (1928) with 0.00012–0.0012 M-fluoride on isolated mammalian hearts, and Chang (1938) with 0.0078 M-fluoride on rabbit auricles. Gottdenker and Rothberger (1935a, b) found depression of the perfused frog heart and the dog heart-lung by fluoride, but on guinea-pig and rabbit auricular strips they usually observed an increase in the amplitude when the fluoride concentration was 0.003 M. Ferrannini (1936) also found fluoride (0.0024–0.0072 M) to increase the force of contraction of rabbit auricular strips. In most work it has been observed that cardiac tissue is able to function surprisingly well in the presence of fluoride at concentrations which should inhibit glycolysis completely. Chang (1938) stated that fluoride, except for a slight depression, had little effect on the mechanic ability of rabbit auricles over a period of two to three hours as long as oxygen was present. Furthermore, he apparently observed no increases in amplitude. In the present work with the same tissue such a concentration of fluoride produced a marked effect. No explanation can be offered for these discrepancies.

With regard to the mechanism of fluoride action, most workers have concluded that depletion of calcium from the medium is not important. However, at least in the present experiments, it is difficult to see how it could fail to be of some importance. The calcium concentration in Tyrode solution is 0.0018 M; if sodium fluoride is added to the medium in a concentration of 0.005 M, there are approximately equivalent amounts of calcium and fluoride to form CaF_2. Since CaF_2 is soluble to the extent of 0.00022 M and since it is very little ionized (solubility product is 3.4×10^{-11}), it would seem likely that a definite fraction of the calcium would be withdrawn from the medium. When more fluoride is added the calcium should be reduced even more. No visible precipitation of CaF_2 was observed, but the walls of the tissue bath slowly became covered with a white layer. Reduction of calcium in the medium leads to a marked depression of the auricles with respect to amplitude, but has no effect on the rate (Webb, 1950). Thus it would seem that the observed depression of rate and the subsequent increase in

amplitude, in the presence of fluoride, must have been due to an action of fluoride other than that of altering the calcium concentration in the medium. As to how much the initial depression of amplitude is due to decreased calcium it is impossible to say.

Since fluoride inhibits cholinesterase, the possibility exists that at least part of the depressant action was due to prevention of destruction of endogenous acetylcholine. However, fluoride was found to give a typical depression of the auricles in the presence of physostigmine (10^{-6}), when the cholinesterase was already inhibited, and also in the presence of atropine (10^{-5}), when the action of acetylcholine was completely prevented. It is thus unlikely that the action of fluoride is related to the acetylcholine equilibrium in the auricles.

Such concentrations of fluoride as were found to have definite actions on the auricles (0.005–0.05 M) should inhibit glycolysis completely. Muscle enolase is usually inhibited 50 per cent by 0.001 M-fluoride and completely by 0.005 M-fluoride. A certain decrease in fluoride concentration would result from combination with calcium, but in the higher concentrations this could not have been very great. We must, therefore, conclude that the auricles can function quite well in the absence of the formation of phosphopyruvate from 2-phosphoglycerate. One possibility is that the auricles used fat for the necessary energy. Another possibility is that the 2-phosphoglycerate was utilized through some other system without the formation of pyruvate. It is interesting to speculate that the secondary increase in amplitude is due to this new source of energy, brought into play because of the enolase block. In rat heart slices the respiration was never depressed more than 42 per cent by fluoride at concentrations as high as 0.1 M (Webb, Saunders, and Thienes, 1949b), so it is evident that in cardiac tissue there is an appreciable fraction of the metabolism insensitive to fluoride.

Fluoride inhibition is reversible, and after the fluoride had been washed out recovery was usually complete within 5–10 minutes. After long exposures there was occasionally some stimulation of the rate above the normal figure during the post-wash recovery stage. It was also noticed that the auricles during this period frequently had difficulty in relaxing and the tone varied correspondingly. In passing, it may be remarked that fluoride (0.03 M) quite effectively diminished the action of adrenaline (10^{-7}–10^{-6}), the stimulation of amplitude being almost abolished while the rate effect remained but was made weaker.

Iodoacetate

Iodoacetate in concentrations between 0.0001–0.001 M is a slowly acting depressant of the rabbit auricle, requiring from three to five minutes for appreciable reduction of the amplitude and from 10 to 20 minutes for a slowing of the rate. At certain stages of the depression, the amplitude was markedly reduced while the rate remained unaffected, but eventually both were depressed almost equally before the auricles stopped. During the later stages of this depression the auricles began to go into contracture and they stopped in systole, a phenomenon that has been invariably observed in work with iodoacetate (Weicker, 1934; Chang, 1938; and others). Although slight depressions produced by low concentrations of iodoacetate acting for short periods of time could be partially reversed by removing the iodoacetate, the usual result was that after the iodoacetate had been washed out the inhibition

proceeded, exactly as if the inhibitor were still present in the medium. This is, of course, what one would expect from considerations of the irreversible cellular reactions of iodoacetate, and it confirms previous results on other tissues and preparations.

Iodoacetate is not a specific inhibitor of glycolysis in mammalian cardiac tissue (Webb, Saunders, and Thienes, 1949b), and even in low concentrations it is likely that other enzyme systems are involved in its action. Not only is the synthesis of ATP diminished, by inhibition of the utilization of pyruvate and fatty acid breakdown products as well as by prevention of glycolysis, but it is likely that iodoacetate interferes with the utilization of ATP, for Buchtal, Deutsch, and Knappeis (1944) have reported that iodoacetate blocked the stimulating effect of ATP on skeletal muscle. It would not be surprising if iodoacetate reacted directly with myosin, since this protein complex possesses a number of sulphhydryl groups important to its functioning. This may explain the observation (Chang, 1938) that there is some ATP present in rabbit hearts that have been stopped by iodoacetate.

Adrenaline produced very little stimulation of the iodoacetate depressed auricle, and occasionally a reversal of the rate effect was observed when the depression was well advanced. In one typical experiment the concentration of iodoacetate was 0.001 M, the exposure time was 35 minutes, and the auricles were markedly depressed (rate −42 per cent, amplitude −96 per cent); adrenaline (10^{-6}) stimulated the amplitude slightly (28 per cent), but the rate was decreased to −75 per cent of the original value. It is not surprising that such auricles would fail to be stimulated by adrenaline, but the reversal of the rate effect was not expected. It is possible that iodo-acetate blocks some step in the series of reactions through which adrenaline normally produces a stimulation of the rate, leaving the systems by which it may depress the rate at higher concentrations relatively unaffected.

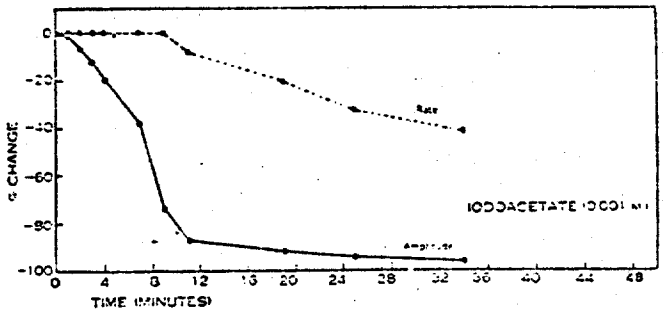

FIG. 9.—The action of iodoacetate on the rabbit auricle.

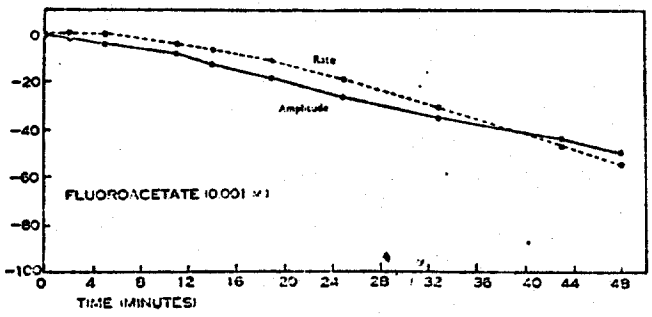

FIG. 10.—The action of fluoroacetate on the rabbit auricle.

Fluoroacetate

Fluoroacetate is the most slowly acting inhibitor of those investigated, depressing the auricles gradually and steadily for a period of two to three hours at a concentration of 0.001 M. It characteristically depressed both rate and amplitude equally, in this respect differing from any other inhibitor used in this study. The metabolic systems involved in impulse discharge and contraction may at some point possess a common path which is attacked by fluoroacetate. At the present time the exact mechanism of action of fluoroacetate is unknown, but it is likely that it inhibits some reaction, or reactions, in the tricarboxylic acid cycle. Accumulation of citrate and acetate during fluoroacetate action has been reported, but the depression of the auricles was not due to these intermediates, as may be seen by comparing the action of fluoroacetate with the actions of these substances. Furthermore, if such were the explanation, we should expect some degree of recovery when fluoroacetate was removed from the bath, but this did not occur. The action of fluoroacetate was quite irreversible. Occasionally the inhibition could be arrested by washing out the inhibitor, but the auricles never showed any tendency to return to their normal rate and amplitude.

Phlorizin

A nearly saturated solution of phlorizin in the medium (0.001M) produced a slight depression of the auricles, the effect on the rate being somewhat greater than on the amplitude, the latter showing some ability to recover spontaneously in the presence of the inhibitor. The mechanism of action of phlorizin at these higher concentrations is not well understood, and therefore it is difficult at the present time to interpret these results or correlate the actions with any particular inhibition of enzymatic systems.

Pyrophosphate

Pyrophosphate at concentrations between 0.002–0.005 M produced a marked depression in the amplitude of the auricles but had little effect on the rate, slight

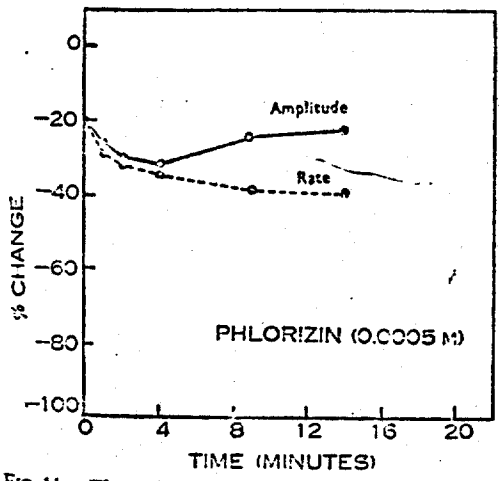

FIG. 11.—The action of phlorizin on the rabbit auricle.

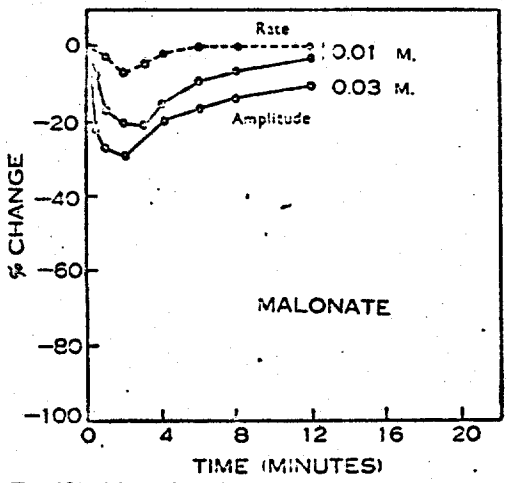

FIG. 12.—The action of malonate on the rabbit auricle.

stimulation occasionally being the only effect observed. Since pyrophosphate combines with calcium and the medium became slightly turbid after its addition, the possibility was considered that the action upon the auricle was due to a decrease of ionized calcium in the medium. If calcium was added to the medium during pyrophosphate depression the amplitude returned to normal, but the increased rate was unaffected. Lack of calcium in the medium leads to depression of the amplitude, but the rate is never stimulated and is occasionally slightly reduced. Recovery of the auricles after low calcium was more rapid than after pyrophosphate depression. We may conclude, therefore, that the effects produced by pyrophosphate were partially due to a lack of ionized calcium, but that the pyrophosphate also had a definite action of its own. Since these actions could not be separated conveniently, the action of pyrophosphate was not studied farther and the curves of its action are not included in the Figures.

Malonate

The rabbit auricle was not strongly depressed by malonate: at high concentrations (0.02–0.03 M) a maximum inhibition of only 30 per cent in rate and amplitude was reached. Furthermore, the auricles spontaneously recovered in the presence of malonate at these high concentrations and returned to normal, or nearly normal, rates and amplitudes after 8–10 minutes. When the malonate was washed from the bath during the period of greatest depression, the recovery was very rapid, requiring only two to three minutes. Forssmann and Lindstén (1946) found that malonate in concentrations between 0.01–0.04 M depressed the ventricular contractions of the perfused rabbit heart but had little effect on the rate. It is remarkable that malonate at these concentrations should have so little depressant action on the auricles, for it is generally believed that malonate at a concentration of 0.005 M completely inhibits succinic dehydrogenase and therefore blocks the tricarboxylic acid cycle, a system upon which the cardiac muscle is thought to depend. Recently it has been reported that somewhat higher malonate concentrations, such as those used here, will prevent the oxidation of oxalacetate in homogenates, this action producing an additional derangement of the tricarboxylic acid cycle (Pardee and Potter, 1949). It seems unlikely that failure to penetrate the muscle cells could have been responsible for this lack of action, inasmuch as exposures of 20–30 minutes to malonate did not lead to any marked alteration of auricular function. The strange behaviour of cardiac cells under the influence of malonate was noted previously on rat heart slices (Webb, Saunders, and Thienes, 1949b). In this preparation malonate produced an initial depression of the respiration, but this was followed by a recovery to values near the normal rate. Furthermore, in the rat heart slices it was known that the succinic dehydrogenase was inhibited, for no rapid increase in oxygen uptake was observed when succinate was added as invariably happened when malonate was absent. Rat heart slices can respire adequately in the presence of high concentrations of malonate, and auricles can function quite normally under these conditions. We must conclude that cardiac tissue can respire and function when this particular step in the tricarboxylic acid cycle is blocked. It may well be that when this block is imposed in an organized cellular system, the cycle can be short-circuited: perhaps under such conditions oxalacetate may be formed directly from α-ketoglutarate, or pyruvate or its activated fragment be taken into the

cycle by combination with something other than oxalacetate. It will be shown later that the small amount of depression observed with malonate is probably not due to the inhibition of succinic dehydrogenase.

Interactions of substrates and inhibitors on the auricles

Experiments were performed to investigate the abilities of various substrates to counteract or antagonize the depressions produced by metabolic inhibitors. Theoretically, if an inhibitor acts specifically on one enzyme in a series of enzymic reactions, the addition of the intermediate substrate that is normally formed by the action of this enzyme should allow at least partial recovery of the functional activity of the tissue. If the inhibitor acts not only on this one enzyme, but upon others as well, the amount of recovery produced by the added intermediate will be roughly proportional to the specificity of the inhibitor effect. The possibility must always be considered that an added substrate, in the presence of an inhibitor, may initiate energy-forming reactions that are not normally important; thus, a stimulation of the auricles by a substrate during an inhibitor depression does not necessarily mean that there is a direct relationship between the inhibitor and the substrate in the cellular metabolism.

The relatively weak effect of malonate on the auricles was puzzling, and attempts were therefore made to determine whether the depression that was produced was related to the commonly accepted action of malonate on succinic dehydrogenase. Malonate prevents the formation of fumarate from succinate, and recovery from malonate depression should, therefore, be observed after the addition of fumarate. If malonate acted simultaneously elsewhere in the tricarboxylic acid cycle this would not necessarily be true. Three types of experiment were performed.

(1) In two experiments the effects of malonate (0.01–0.03 M) were first determined, and these were compared with the effects of adding malonate and fumarate (0.001–0.01 M) together. No modification of the action by fumarate was observed. In a typical experiment the average maximum depression of amplitude produced by malonate alone was 25.7 per cent; when both malonate and fumarate were added together the depression was 27.7 per cent. The changes in rate were likewise comparable.

(2) In the second type of experiment fumarate (0.001–0.01 M) was added to auricles already depressed by malonate (0.02 M). No stimulating effect was observed; in fact, an additional depression was usually seen.

(3) In the third type of experiment the auricles were allowed to remain in contact with fumarate (0.005 M) for five minutes before the addition of the malonate (0.01 M) in order to ensure penetration of the fumarate. Again no change in the response to malonate was observed.

It therefore seems likely that the malonate depression is not due solely to inhibition of succinic dehydrogenase. It is more reasonable, I believe, to assume that malonate at such concentrations exerts a toxic action on the cell surface, a depression such as is seen with any of such substances under similar conditions. Acetate (0.001 M) and pyruvate (0.005 M) were also unable to counteract the depression produced by malonate (0.02 M), only additional depression being observed.

The depression produced by fluoride (0.005–0.02 M) was not counteracted by succinate (0.001 M), acetate (0.001 M), or citrate (0.001 M). However, pyruvate (0.005 M) showed some ability to stimulate the rate of the auricle in the presence of fluoride, but the amplitude was usually unaffected. This is in harmony with most of the results obtained with pyruvate, namely that it acts more particularly upon the mechanisms involved in rate than upon those involved in the amplitude of contraction. Complete antagonism of fluoride with pyruvate was never observed.

Iodoacetate, like fluoride, is generally supposed to act predominantly on the glycolytic stages of metabolism, inhibiting the formation of pyruvate from glycogen or glucose. However, there are reasons for supposing that in cardiac tissue the action is not specific. The heart muscle presumably obtains energy from both glycolytic and post-glycolytic phases of metabolism, although a relatively small amount from the former (approximately 15–20 per cent of the total). If iodoacetate acted only on glycolysis, the addition of pyruvate should restore the activity of the heart muscle to about 80 per cent of its normal value, only the energy from the glycolysis now being lost to the tissues. Three experiments were performed to test this.

(1) In the first experiment the concentration of iodoacetate was quite high (0.001 M), the auricles had been in contact with the inhibitor for 15 minutes, and the resulting depression was severe (rate −40 per cent, amplitude −95 per cent). Addition of pyruvate (0.01 M) gave no stimulation, the rate and amplitude continuing to decrease and contracture to develop. After the auricles were completely stopped at 30 minutes the addition of pyruvate was without effect.

(2) In the second experiment the concentration of iodoacetate was lower (0.0002 M), the time of exposure to the inhibitor was 25 minutes, and the auricles were very depressed (rate −83 per cent, amplitude −87 per cent). The addition of pyruvate (0.005 M) slightly stimulated the rate but had no effect on the amplitude. The normal rate before iodoacetate was added was 72, the iodoacetate had reduced it to 12; pyruvate increased the rate to 18 in two minutes and to 24 in four minutes. Later in the depression pyruvate had no effect at all.

(3) In the third experiment the iodoacetate (0.0002 M) was allowed to act for 15 minutes, the auricles being moderately depressed (rate −19 per cent, amplitude −53 per cent). Pyruvate (0.005 M) slightly stimulated both rate and amplitude, the rate increasing by 15 per cent in two minutes and the amplitude increasing by 32 per cent within 30 seconds; these were the maximum effects and were only temporary, the depression of the auricles proceeding as if the pyruvate had not been present. Later in the depression pyruvate was added at both 0.001 M and 0.01 M without any effect.

One may conclude that pyruvate is only partially able to counteract the depression produced by iodoacetate, and that the higher the concentration of iodoacetate and the longer its action, the less recovery may be observed. These experiments confirm previous conclusions that iodoacetate is not a specific poison of the glycolytic phase of metabolism in mammalian cardiac muscle. Acetate (0.001–0.01 M) showed no ability to counteract the iodoacetate depression.

Depression of the auricles produced by fluoroacetate was partially antagonized by pyruvate. This was demonstrated in two experiments.

(1) In the first experiment the concentration of fluoroacetate was 0.001 M, the time of exposure was two hours, and the depression was very marked (rate −79 per cent, amplitude −83 per cent). Pyruvate (0.01 M) increased the rate by 133 per cent and the amplitude by 18 per cent within one minute, the rate returning to half its normal level and the amplitude to only 20 per cent of its normal value. The addition of pyruvate at the same concentration 35 minutes later produced even greater stimulation, the rate increasing by 200 per cent and the amplitude by 126 per cent in two minutes.

(2) In the second experiment the fluoroacetate (0.001 M) was allowed to act for only 25 minutes and the auricles were only moderately depressed (rate −24 per cent, amplitude −25 per cent). Pyruvate (0.005 M) gave some stimulation, increasing the rate by 8 per cent and the amplitude by 4 per cent within one minute. After an exposure of 50 minutes, when the auricles were more depressed (rate −41 per cent, amplitude −62 per cent), pyruvate at the same concentration stimulated more successfully, the rate increasing by 40 per cent and the amplitude by 21 per cent. The effect on the amplitude was quite temporary and after three or four minutes the auricles were again being progressively inhibited, but the rate effect remained for a much longer time.

Discussion

Each metabolic inhibitor used in the present investigation has been found to exert a unique and characteristic action upon the auricle, the effects differing from one another with respect to (*a*) the concentration-action curves, (*b*) the time course of action, (*c*) the relative actions on rate, amplitude of contraction, and muscular relaxation, (*d*) the ability of the cardiac muscle to recover spontaneously in the presence of the inhibitor, and (*e*) the ability to recover after the inhibitor has been removed. Each inhibitor can be distinguished from the rest by its action on the rabbit auricle. The problem, however, is to determine whether any useful information on the mechanisms of cardiac function may be derived from such data in conjunction with the results on substrates, substrate depletion, and anoxia.

The auricles may be stopped in either diastole (state of relaxation) or systole (state of contraction). Substances which arrest the auricles rapidly always stop them in a state of relaxation. The process of contracture, by which the auricles stop in systole, requires time for its development and is not observed early in the action of any substance. There are substances which will rapidly arrest the auricles in diastole and, with continuing action, will induce contracture, the end result being systolic arrest. In the present experiments with rabbit auricles, the following have been found to lead to contracture: iodoacetate, fluoroacetate, fluoride, cyanide, anoxia, and substrate depletion. All of these would be expected to produce a state of reduced metabolic activity with a decrease in the amount of energy available to the muscle. This inhibition of the flow of energy would result in a gradual loss of ATP (and other substances containing high-energy phosphate bonds), such having been observed in the past under similar conditions. Simultaneously with the depletion of ATP the cardiac muscle becomes less and less able to relax, and when the ATP level becomes sufficiently low the muscle goes into complete contracture and

will beat no longer. The ATP concentration need not drop to zero for this to occur. This conception is in harmony with the views of Szent-Györgyi (1947) on skeletal muscle, ATP being required for the relaxation of the muscle fibrils as well as for their contractile activity. The auricular muscle is in a low-energy state when it is contracted and in a high-energy state when it is relaxed. Adding glucose or pyruvate to a muscle in contracture would provide some ATP and the muscle would be able to relax and beat again.

Let us consider auricles that have been stopped in the relaxed state of diastole by some metabolic inhibitor. Why does the muscle not contract? There is certainly present a sufficient concentration of ATP. There is nothing fundamentally wrong with the contractile mechanisms, for the muscle can be made to contract by electrical stimulation, and it will recover from such contractions for a period of time. The initiating reaction for contraction must be depressed. The functioning of cardiac muscle involves not only the mechanisms of contraction and relaxation, but also processes that are rhythmic in nature and serve to stimulate these contractions. Normal contraction is induced by a wave of depolarization (accompanied simultaneously by other changes) which sweeps over the muscle and in some manner causes the actomyosin to be transformed into its low-energy state. This stimulating process can be roughly divided into three phases: the discharge of the impulse from the pacemaker cells, the conduction of this impulse, and the excitatory reaction by which the actomyosin is affected by the impulse. These process are all dependent on metabolic reactions for their energy. Maintenance and restoration of the cellular membrane potentials require energy, and further energy may be required for the synthesis and distribution of substances involved in these processes. It is likely that these excitatory mechanisms are inhibited in auricles that have been arrested in diastole. Occasionally a substance will stop the heart by depressing the discharge of impulses from the pacemaker cells, but usually it would seem to be a matter of depression of conduction or of the excitatory reaction (the process by which the actomyosin is stimulated by the conducted impulse will be here termed the "excitatory reaction"). In many cases the amplitude of the auricles was gradually depressed until arrest occurred; immediately before arrest the rate was depressed relatively little. Observation of such auricles leads one to the conclusion that the pacemaker cells are still discharging after the auricles have ceased to beat, the failure being due to disturbances in conduction or the excitatory reaction. If such is the case, it lends support to the view that partial inhibition of the amplitude is due to the failure of a certain fraction of the contractile units to react, the contraction of each unit being all-or-none in nature. Progressive inhibition of the excitatory reaction would lead to a gradual decrease in the number of units responding to the conducted impulse, and this in turn would lead to a progressive inhibition of the amplitude of contraction. Conduction and the excitatory reaction may be more sensitive than the processes of contraction and relaxation to metabolic inhibitors and anoxia because they do not possess a store of ATP, most of this substance being found in the actomyosin or in the enzymes involved in phosphate transfer.

When the amplitude of contraction of cardiac tissue is reduced to some fraction of its normal value (no change in the degree of relaxation having taken place), we may interpret this in two general ways. Either there is a smaller fraction of

all-or-none units contracting, or each unit in the muscle is contracting proportionately less. The solution to this problem is fundamental to cardiac physiology and pharmacology. Does cyanide, for example, when it inhibits the auricular amplitude by 20 per cent, prevent completely the contractions of 20 per cent of the contractile units, the remaining units contracting normally, or does it reduce the contraction of each unit an average of 20 per cent? This is not merely the old question of whether the concentration-action curve is an expression of the action of a substance on cellular processes or whether it is derived from the distribution of susceptibilities of the individual cells. A depressant may inhibit the excitatory reaction either by raising the threshold of response of the contractile units or by reducing the excitatory state that is initiated by the passage of the impulse. In the former case we should be measuring a distribution of susceptibilities, but in the latter case it would be a matter of a true action on a cellular process. There is no *a priori* reason why both actions may not be of importance, depending on the substance that is producing the depression. It should also be pointed out that, although the contractile units may normally behave in an all-or-none manner, it is entirely possible that various conditions may modify the degree of contraction.

How must we interpret the changes in amplitude brought about by the action of these metabolically important substances on the rabbit auricle? The results of the present investigation can be best explained by assuming that these substances modify the excitatory reaction and not the contractile mechanisms directly. Only when the cardiac muscle begins to go into contracture is the contractile mechanism itself altered. If this is the correct interpretation, it would seem that our conceptions of the actions of substances on cardiac muscle must be modified. Such an interpretation would also apply to the actions of drugs on the heart.

Throughout this investigation it was found that the changes in rate and amplitude of the auricles were relatively independent of each other. An increase or decrease in the amplitude had no particular effect on the rate, nor did changes in the rate, within limits, affect the amplitude. Quite frequently one would remain constant during a marked change in the other, and occasionally they would change in the opposite directions. The fundamental energy-forming enzymatic reactions may be quite similar in the processes that control rate and amplitude, but the paths of energy flow and utilization are different. It is true that most substances change the rate and amplitude in the same direction. This is perhaps more readily understandable when we consider changes in amplitude as arising, not from alterations in the contractile mechanisms, but from modifications of some cellular surface phenomenon that is involved in the excitatory reaction, such a surface state being related to the cellular membrane changes occurring in the pacemaker cells. In whatever way we consider the cyclical discharge of impulses, we must assume that there is the development of a depolarized state and that this is propagated over the cardiac muscle, this being responsible, directly or indirectly, for the excitatory reaction and the contraction of the myofibrils that lie in the region over which the impulse is passing. We can thus see a possible connexion between rate and amplitude, a relationship that implies no necessary simultaneous behaviour, but which, in the majority of cases, will lead to parallel

behaviour with respect to direction of change. However, the magnitudes of the changes in rate and amplitude need bear no relationship to one another.

Development of contracture was always a late process, following earlier alterations in the auricular rate and amplitude. We have supposed that this was due to a greater reserve of ATP or other substances containing high-energy bonds. It is indeed possible that the membrane phenomena of impulse discharge, conduction, and the excitatory reaction do not utilize ATP or similar substances, but that the cyclical processes involved are more directly dependent on the oxidative sequence of enzymatic reactions. Most of the metabolic inhibitors investigated, as well as substrate depletion and anoxia, depressed the amplitude more than the rate. This might be explained by assuming that the rate processes require less energy than those involved in the excitatory reaction, but this can hardly be the entire answer since there are instances in which the rate is depressed as much as the amplitude. The metabolic systems involved in the rate and amplitude may also be assumed to be somewhat different, and this is quite likely from a consideration of the characteristic actions of the various inhibitors, but it would explain only the differences in behaviour and not the basic fact that the rate is more resistant to change than the amplitude. Another explanation might be that the rate and amplitude mechanisms alter their functional level to different degrees in response to an equivalent change in some common factor. For example, if we assume that both the rate of discharge and the effectiveness of the impulse on the contractile units depend on changes in the degree of electrical polarization of the cell membrane, it is conceivable that a depression of the resting potential or a depression of the rate of repolarization would inhibit both processes. However, owing to the particular circumstances, the number of units reacting would be depressed more readily than the discharge rate. The excitatory reaction is probably quite delicately adjusted to the changes occurring at or near the cellular surface. This delicate balance assures a ready adaptability of the cardiac muscle.

We might expect, for the same reasons, that glucose and pyruvate would stimulate the amplitude more than the rate in substrate depleted auricles. This was found to be true for glucose, but pyruvate acted more particularly on the rate. Quantitative comparisons are complicated by the third action of these substrates, the stimulation of relaxation, an action somewhat more readily produced by glucose than by pyruvate. However, it was clear that pyruvate, both in substrate depleted auricles and in those poisoned by inhibitors, stimulated the rate more effectively than the amplitude and more effectively than it produced relaxation of the muscle. From this it might be concluded that the oxidations in the tricarboxylic acid cycle are the main source of energy for the rate processes. Supplying glucose does not lead to a sufficiently rapid formation of pyruvate, and therefore giving pyruvate directly has a greater stimulating effect. Why, however, is pyruvate less effective than glucose in stimulating the amplitude? The energy derived from the breakdown of glucose to pyruvate must be of greater importance to the processes involved in the contraction and relaxation of the muscle than it is to the rate processes. It is doubtful whether we are measuring the effect of either substrate on the excitatory reaction under such circumstances, the stimulation of ATP formation being the primary action in increasing activity of contraction. According to Szent-Györgyi (1947) the actomyosin requires so much ATP to

be active at all, aside from the ATP that is utilized for relaxation. Since ATP can be formed from energy derived from the breakdown of glucose to pyruvate it is not so surprising that glucose is somewhat more effective than pyruvate in this respect. However, in the cellular membrane processes of impulse discharge and the excitatory reaction the conditions are different; as we have seen, it is possible that these mechanisms do not require ATP and may operate directly from the oxidative systems, especially the tricarboxylic acid cycle. Since the tricarboxylic acid cycle can continue to operate in the metabolism of fat, the observation that the rate is more steady during inhibition of glycolysis (iodoacetate) than it is during inhibition of the tricarboxylic acid cycle (fluoroacetate) is explained. There remain, however, a number of problems—for example, the anomalous behaviours of malonate and fluoride—and these ideas are presented only as a working hypothesis.

The metabolic activities of contracted and relaxed cardiac muscle may be different. The metabolism may occur in cycles, in phase with contraction and relaxation, so that the conception of the metabolism as a steady state would be erroneous. Deductions based on steady state conditions would not necessarily be applicable, nor would arguments based on limiting reactions always be valid. This must be kept in mind in considerations of the action of metabolic substrates and inhibitors on the heart. Determinations of the ability of heart slices to oxidize various substrates may give information on only one phase of the cycle; it is likely that such slices are in a state of contracture. The same reasoning would be applicable to the actions of inhibitors. Substrates that are well oxidized by the contracted muscle may produce no stimulation of the normally beating auricle because their oxidation occurs at phases of the cycle where their energy cannot be utilized; only when the cardiac muscle has become abnormal, when these cyclical processes have become distorted, or when the muscle has gone into contracture will these substrates be oxidized with the production of utilizable energy. Similar metabolic cycles of activity probably occur in the pacemaker cells during rhythmic discharge of impulses and near the surfaces of the muscle cells as the propagated impulses pass.

Furthermore, one is not justified in assuming that there is a general synthesis and supply of ATP upon which all the processes in the cardiac cell must draw. ATP is probably used in only certain functional mechanisms and is synthesized in these particular regions. The concentration of ATP as determined by analysis of the entire muscle can seldom be correlated with the functional activity of the tissue for this reason. When one considers the various processes which control the beat of cardiac tissue, the different metabolic requirements of these processes, the spatial relationships, and the cyclical nature of the metabolism, it is easy to understand why it is so difficult to correlate analyses for substances containing high-energy bonds and for other metabolic intermediates with the level of activity of the cardiac muscle.

Summary

1. The actions of thirteen metabolic substrates and intermediates on the rabbit auricle have been investigated, and it was found that they all depress the normal auricle, with regard to both rate and amplitude, except citrate which at higher concentrations stimulated the rate.

2. The behaviour of the beating auricles during depletion of substrate was observed and the abilities of various substrates to revive the auricles have been determined. It was found that only glucose and pyruvate produced marked effects; glucose produced a more marked effect on muscular relaxation, and pyruvate acted particularly upon the rate.

3. The actions of eight metabolic inhibitors on the rabbit auricle have been investigated and each inhibitor was found to produce a characteristic effect. The actions of these inhibitors on the functioning of the auricles does not always parallel their commonly accepted actions on enzymatic systems, and it is concluded that unknown pathways of metabolism exist in cardiac muscle.

4. The abilities of substrates to revive auricles that have been depressed by inhibitors was studied, and the bearing of these results on the specificity of inhibitor action and the nature of myocardial enzyme systems are discussed.

5. None of the observed actions of metabolic substrates and inhibitors are due to an action on the acetylcholine equilibrium in the auricular muscle, because the presence of atropine did not modify them.

6. The mechanisms by which the auricles can be stopped in diastole or systole are discussed in the light of the information obtained on the actions of substrates and inhibitors.

7. It is questioned whether changes in amplitude are directly related to contractile mechanisms. The results obtained could be best explained by assuming that the actions of the substances investigated were upon the mechanisms by which the propagated impulse stimulates the actomyosin to contract into its low-energy state. This important phase of the cardiac functioning is termed the "excitatory reaction." Depression of this excitatory reaction, either by reducing the excitatory state at the cellular membrane or by increasing the stimulation threshold of the contractile units, leads to a reduced number of units contracting and consequently to a reduced amplitude.

8. Changes in rate, amplitude, and degree of relaxation were shown to be relatively independent of each other. From the actions of substrates and inhibitors, as well as substrate depletion and anoxia, on the auricles, an attempt was made to correlate certain phases of metabolism with the mechanisms involved in rate, amplitude, and relaxation. It was concluded that contraction and relaxation are dependent on a certain supply of ATP but that the rhythmic discharge of impulses and the excitatory reaction are more directly dependent on the oxidative reactions of metabolism, particularly the tricarboxylic acid cycle.

9. Some general problems in the correlation of cardiac metabolism with cardiac function are discussed.

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FATTY ACID METABOLISM. THE MECHANISM OF KETONE BODY SYNTHESIS FROM FATTY ACIDS, WITH ISOTOPIC CARBON AS TRACER*

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In a recent communication (1), results were reported of a study of the breakdown of *n*-octanoic acid labeled by incorporation of an excess of the heavy carbon isotope, C^{13}, in the carboxyl group. Proof was offered for a mechanism of ketone body formation involving the random coupling of identical 2-carbon units formed by β oxidation of the octanoic acid. The present paper is concerned with a more detailed description of these experiments and a discussion of the significance of the results obtained to the general question of the oxidation pathways of fatty acids.

EXPERIMENTAL

Preparation of Carboxyl-Labeled n-Octanoic Acid—Carbon dioxide was prepared by the oxidation of 0.49 gm. (0.01 mole) of isotopic sodium cyanide with aqueous potassium permanganate. The CO_2 was released by means of sulfuric acid into a high vacuum system, where it was purified and dried by repeated sublimation at −80° and condensation at −200°. Analysis in the mass spectrometer gave a C^{13} content of 5.51 per cent.[1] The CO_2 was then condensed into a highly evacuated flask containing an excess of *n*-heptylmagnesium bromide in ether. After several hours were allowed for the reaction to go to completion, the reaction mixture was decomposed with dilute sulfuric acid and distilled with steam. The distillate was extracted with ether, the ether extract was shaken with dilute sodium hydroxide, and the aqueous solution of sodium octanoate acidified and re-extracted with ether. The extract was then dried over anhydrous sodium sulfate and the ether evaporated. The residual colorless oil weighed 1.175 gm., representing a yield of 81 per cent. A Duclaux distillation revealed no perceptible volatile acid other than *n*-octanoic. Neutralization equivalent, observed, 143.0; calculated, 144.2.

* With the technical assistance of Mary Cammaroti.

[1] The C^{13} content is expressed as atoms per cent C^{13} calculated from the equation

$$\text{Atoms \% } C^{13} = \frac{\text{moles } C^{13}}{\text{moles } C^{12} + \text{moles } C^{13}} \times 100$$

The normal C^{13} complement for our instrument was found to be 1.01 per cent.

A specimen of this acid was oxidized according to the wet combustion method of Van Slyke and Folch (3). The resultant CO_2 had a C^{13} content of 1.59 per cent, corresponding to a C^{13} content for the carboxyl carbon of $(1.59 \times 8) - (1.01 \times 7) = 5.41$ per cent, in excellent agreement with the value of 5.51 per cent found for the C^{13} content of the original CO_2.

Incubation with Liver Slices—Liver slices of 0.4 mm. thickness from rats fasted 24 hours were shaken for 2 hours at 37.5° with 30 ml. of a 0.01 M sodium octanoate solution in a phosphate-buffered Ringer's solution. The composition of the medium was as follows: sodium octanoate 0.010 M, NaCl 0.051 M, PO_4 buffer, pH 7.4, 0.010 M, $CaCl_2$ 0.001 M, KCl 0.002 M. The reaction was carried out in Warburg type vessels of 125 ml. capacity in an atmosphere of oxygen, with 0.5 ml. of 2 N H_2SO_4 in the side cup and filter paper soaked with 0.5 ml. of 15 N NaOH in the central well. The uptake of oxygen was measured by an attached gas burette. At the end of the incubation period the acid, sufficient to bring the pH of the medium to 3.5, was tipped in from the side cup and shaking continued an additional 15 minutes to allow complete absorption of the liberated CO_2.

Determination of Respiratory CO_2—The alkali-soaked filter paper in the central well was quickly transferred to another Warburg vessel containing 20 ml. of CO_2-free water, the air was swept out with a stream of CO_2-free nitrogen, and, after equilibration, the CO_2 was liberated by acid tipped in from the side cup. The evolved CO_2 was measured volumetrically. The gases were then swept, by means of nitrogen, into an absorption tube containing barium hydroxide, from which the CO_2 was ultimately liberated by acid into an evacuated storage tube for determination of the C^{13} content.

Decomposition of Acetoacetic Acid and Isolation of Acetone and CO_2 Therefrom—The solution was decanted from the liver slices into a 50 ml. centrifuge tube, the slices were washed successively with small portions of dilute alkali and water to remove any adsorbed fatty acid, and the washings added to the solution. The soluble proteins and sugar were removed by the addition of 1 ml. of 25 per cent copper sulfate, followed by sufficient solid calcium hydroxide for alkalinity. After standing ½ hour, the mixture was centrifuged, and the clear supernatant liquid refluxed with mercuric sulfate according to the procedure of Van Slyke (4). The CO_2 liberated by the decomposition of the acetoacetic acid was swept, by a stream of nitrogen, into barium hydroxide solution, from which it was liberated by acid into an evacuated storage tube for determination of the C^{13} content. The precipitated acetone-mercury complex was transferred to a weighed, sintered glass funnel, washed thoroughly, and dried at 110°. It was then oxidized by the wet combustion method of Van Slyke and Folch for determination of the C^{13} content.

The filtrate from the acetone precipitation was then treated with di-

chromate according to Van Slyke (4) fo acid, and the acetone and CO_2 isolated tions were made on the CO_2 evolved du of acetone-mercury complex formed wa

Recovery of Residual Fatty Acids—T ml. and the volatile acids determined Duclaux distillations were carried out o parison with the original octanoic acid.

Isotope Analysis—The C^{13} determina mercial mass spectrometer manufactur Corporation of Pasadena, California.

TABLE

Distribution of C^{13} in Products of Incubation o Liver Slices from

Time, 2 hours; temperature, 37.5°.

	Experiment 1		
Tissue, gm.	1.25		
Volume of medium, ml.	30		
	mg	per cent C^{13}	
Octanoic acid utilized		1.59	0.
O_2 consumed	0.219		0.
CO_2 evolved in respiration		1.41	0.
Acetoacetic acid			
Acetone	0.0383	1.33	0.
Carboxyl		1.80	
Hydroxybutyric acid			
Acetone	0.0162		0.
Carboxyl			

been corrected for the 0.01 per cent of contributing to the mass 45 peak as CO^{13}

Results

The results of four experiments in w acid was incubated for 2 hours with liver in Table I. The mean C^{13} content of the acid is 1.32 ± 0.02 atom per cent, repres over the normal complement of 1.01 per carboxyl group of the acetoacetic acid co an excess of 0.78 per cent.

Location of C^{13} within Acetone Molecule— C^{13} within the acetone the remaining D

d according to the wet combustion. The resultant CO_2 had a C^{13} conten content for the carboxyl carbon o nt, in excellent agreement with the ontent of the original CO_2.

ices of 0.4 mm. thickness from rats rs at 37.5° with 50 ml. of a 0.01 x te-buffered Ringer's solution. The ollows: sodium octanoate 0.010 M, 10 M, $CaCl_2$ 0.001 M, KCl 0.002 M. g type vessels of 125 ml. capacity in . of 2 x H_2SO_4 in the side cup and x NaOH in the central well. The at d gas burette. At the end ien ring the pH of the medium nd shaking continued an additional of the liberated CO_2.

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nd Isolation of Acetone and CO_2 from the liver slices into a 50 ml. successively with small portions of sorbed fatty acid, and the washings oteins and sugar were removed by pper sulfate, followed by sufficient After standing ½ hour, the mixture tant liquid refluxed with mercuric Van Slyke (4). The CO_2 liberated c acid was swept, by a stream of m, from which it was liberated by determination of the C^{13} content. plex was transferred to a weighed, r, dried at 110°. It was then of Slyke and Folch for deter-

itation was then treated with di-

nate according to Van Slyke (4) for the oxidation of β-hydroxybutyric d, and the acetone and CO_2 isolated as described above. C^{13} determina- ns were made on the CO_2 evolved during the oxidation, but the amount acetone-mercury complex formed was too low for C^{13} determinations.

Recovery of Residual Fatty Acids—The final filtrate was diluted to 200 ml. and the volatile acids determined by the method of Friedemann (5). Dudaux distillations were carried out on the residual fatty acids for com- parison with the original octanoic acid.

Isotope Analysis—The C^{13} determinations were carried out with a com- mercial mass spectrometer manufactured by the Consolidated Engineering Corporation of Pasadena, California. The C^{13} values given herein have

TABLE I

Distribution of C^{13} in Products of Incubation of 0.01 M Carboxyl-Labeled Octanoate with Liver Slices from Fasted Rats

Time, 2 hours; temperature, 37.5°.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Tissue, gm. / Volume of medium, ml.	1.25 / 30		1.25 / 30		2.50 / 60		2.50 / 60	
	mM	per cent C^{13}	mM	per cent C^{13}	mM	per cent C^{13}	mM	per cent C^{13}
Octanoic acid utilized		1.59	0.207	1.59	0.389	1.59	0.309	1.59
O_2 consumed	0.219		0.242		0.338		0.283	
CO_2 evolved in respiration		1.41	0.143	1.34	0.221	1.35	0.161	1.39
Acetoacetic acid								
Acetone	0.0383	1.33	0.0787	1.34	0.0579	1.30	0.0755	1.33
Carboxyl		1.80		1.80	0.0330	1.78	0.0659	1.85
Hydroxybutyric acid								
Acetone	0.0162		0.0143		0.0291		0.0376	
Carboxyl				1.22			0.1623	1.15

been corrected for the 0.04 per cent of O^{17} naturally present in oxygen contributing to the mass 45 peak as $CO^{16}O^{17}$.

Results

The results of four experiments in which carboxyl-labeled *n*-octanoic acid was incubated for 2 hours with liver slices from fasted rats are shown in Table I. The mean C^{13} content of the acetone moiety of the acetoacetic acid is 1.32 ± 0.02 atom per cent, representing an excess of 0.28 per cent over the normal complement of 1.04 per cent. The CO_2 derived from the carboxyl group of the acetoacetic acid contained 1.82 ± 0.03 per cent C^{13}, an excess of 0.78 per cent.

Location of C^{13} within Acetone Molecule—To ascertain the position of the C^{13} within the acetone the remaining Denigès mercury-acetone complex

from the four experiments was pooled, giving in all 10.2 mg., representing 2.01 mg. of acetone. It was dissolved in 2 ml. of 6 N HCl and the acetone distilled at room temperature under reduced pressure into a cooled mixture of 2 ml. of 15 N NaOH and 2 ml. of 0.1 N iodine solution. After warming to room temperature, the mixture was acidified. The iodoform was separated by centrifugation, washed thoroughly, and oxidized to CO_2 by the wet combustion procedure of Van Slyke and Folch. The CO_2 thus obtained had only the normal complement of C^{13}, 1.04 per cent. Since the iodoform in this procedure is derived from the methyl carbons, it is clear that the excess C^{13} of the acetone is exclusively in the carbonyl carbon. Inasmuch as the mean C^{13} content of the entire acetone molecule is 1.32, an excess of 0.28 per cent, the C^{13} excess of the carbonyl carbon is (3 × 0.28) or 0.84 per cent.

Respiratory CO_2—The mean value for the C^{13} content of the respiratory CO_2 was 1.37 ± 0.03 per cent, representing an excess of 0.33 per cent. Since the octanoic acid contained 1.59 per cent C^{13}, or an excess of 0.55 per cent, the average fraction of the respiratory CO_2 derived by complete oxidation of the substrate is 0.33/0.55 or 60 per cent of the total respiratory CO_2.

Disappearance of Octanoic Acid—In three of four experiments shown in Table I determinations were made of the amount of *n*-octanoic acid which disappeared during incubation. Of 0.207, 0.389, and 0.309 mM of octanoic acid utilized in these experiments only 0.0930, 0.0873, and 0.1131 mM respectively of total ketone bodies (as acetone) were recovered. Thus, of the octanoic acid disappearing, only 22, 11, and 18 per cent respectively can be accounted for as ketone bodies. Because of this surprising finding the method of recovery of the octanoic acid was carefully investigated. The residual acid was generally recovered after the successive steps of deproteinization, precipitation of acetone, and oxidation of β-hydroxybutyric acid. Preliminary experiments with known quantities of acetoacetic, β-hydroxybutyric, and *n*-octanoic acids of the same order as is dealt with in these experiments gave quantitative recoveries of each component. However, in order to rule out any possibility of loss of octanoic acid by any of these procedures, in Experiments 3 and 4 aliquots of the solution after incubation were acidified and distilled directly. The octanoic acid recovered by this procedure was not significantly greater than that obtained after deproteinization and ketone body isolation. The possibility that octanoic acid may be bound by the tissue was also investigated. A buffered 0.01 M solution of octanoic acid was divided into two equal parts, one of which was distilled directly, the other shaken with 1.25 gm. of liver slices and then distilled. The recovery of acid was quantitative in both

ances; hence there is no significant a
tissues.

Volatile Fatty Acids—The Duclaux con
and the voltaile acids obtained by direc
compared in Table II. There is indicate
of a more volatile acid accompanying
butyric and hexanoic acids can be detecte
tions, there is little likelihood they are
That the acid is possibly acetic acid is
Duclaux constants of the volatile acids
consisting of 90 per cent octanoic and 10
The constants are practically identical.

TABLE II
Duclaux Constants of

	12.5 per cen
n-Octanoic acid	88.0
Carboxyl-labeled *n*-octanoic acid	86.1
Volatile acids, Experiment 4	79.5
90% octanoic acid-10% acetic acid	79.0
Acetic acid	14.7

DISCUSSIO

Mechanism of Ketone Body Synthesis—T
(6, 7) assumes that successive β oxidation
the last 4 carbon atoms, in the form of buty
the ketone bodies. As originally conceive
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result of β oxidation (8–10), and the format
of more than 1 molecule of ketone body p
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posed. To account for the high yields
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the r shaken with 1.25 gm. of liver
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instances; hence there is no significant absorption of octanoic acid by the tissues.

Volatile Fatty Acids—The Duclaux constants for the initial octanoic acid and the voltaile acids obtained by direct distillation after incubation are compared in Table II. There is indicated the presence of a small amount of a more volatile acid accompanying the octanoic acid. Inasmuch as butyric and hexanoic acids can be detected by odor in very low concentrations, there is little likelihood they are present in significant amounts. That the acid is possibly acetic acid is indicated by comparison of the Duclaux constants of the volatile acids with those of a synthetic mixture consisting of 90 per cent octanoic and 10 per cent acetic acid (Table II). The constants are practically identical.

TABLE II

Duclaux Constants of Fatty Acids

	Total volume distilled				
	12.5 per cent	25.0 per cent	37.5 per cent	50.0 per cent	75.0 per cent
	Per cent of total acidity in distillate				
n-Octanoic acid	88.0	96.5	97.9	98.4	100.0
Carboxyl-labeled *n*-octanoic acid	86.1	97.0	98.2	98.8	100.0
Volatile acids, Experiment 4	79.5	91.0	94.3	96.1	100.0
90% octanoic acid-10% acetic acid	79.0	91.1	93.6	95.8	100.0
Acetic acid	14.7	30.1	46.4	63.9	100.0

DISCUSSION

Mechanism of Ketone Body Synthesis—The classical theory of β oxidation (6, 7) assumes that successive β oxidation along the fatty acid chain occurs, the last 4 carbon atoms, in the form of butyric acid, being oxidized directly to the ketone bodies. As originally conceived, this theory failed to account for the absence of acetic acid, which would be expected in large amounts as a result of β oxidation (8–10), and the formation, under certain circumstances, of more than 1 molecule of ketone body per molecule of fatty acid catabolized (11). As a result, several modifications of this theory have been proposed. To account for the high yields of ketone body by liver slices Jowett and Quastel (11) revived the hypothesis of Hurtley (8) that oxidation occurs at alternate carbon atoms throughout the fatty acid chain prior to splitting into units of 4 carbon atoms. Known as the theory of multiple alternate oxidation, it has been accepted widely (10–12).

Recently MacKay (13) has suggested that the ketone bodies may be formed by condensation of acetic acid (or some other 2-carbon intermediary)

resulting from β oxidation of the fatty acid. Although no experimental evidence was offered for this hypothesis, it is consistent with the known facts on which are based the earlier theories, and it explains certain findings which cannot be reconciled with the theory of multiple alternate oxidation: namely, (*a*) the formation of more ketone bodies from hexanoic than from butyric acid (12), and (*b*) the formation of ketone bodies from valeric acid, 3 carbon atoms of which are known to form glycogen (14, 15).

The essential correctness of the principle of β oxidation was conclusively demonstrated by Schoenheimer who found, with deuterium-containing fatty acids, the mutual interconversion *in vivo* of palmitic and stearic acids (16).

The presence of excess C^{13} in the acetone portion of acetoacetic acid formed by the breakdown of carboxyl-labeled *n*-octanoic acid is proof that a redistribution of the carbon atoms occurred during this process, a result which is inconsistent with either the classical theory of β oxidation or the multiple alternate oxidation hypothesis. Moreover, the equal distribution

TABLE III

Comparison of Observed Values of C^{13} Distribution in Acetoacetic Acid Predicted by Various Theories of Ketone Body Formation

	Atom per cent C^{13}, excess	
	Carbonyl	Carboxyl
Observed	0.84	0.83
β oxidation-condensation theory	1.10	1.10
Multiple alternate oxidation theory	0	2.20
Classical β oxidation theory	0	0

of the excess C^{13} between the carbonyl and carboxyl carbons of acetoacetic acid and the absence of excess C^{13} from the methyl and methylene carbons are consistent only with a mechanism involving the random coupling of identical 2-carbon units. The observed distribution of the excess C^{13} between the carbonyl and carboxyl carbons, compared with the values calculated for the three theories discussed above, is shown in Table III. The correspondence between the observed values and those predicted by the β oxidation-condensation mechanism is actually better than that indicated in Table III when it is recalled that in the absence of a substrate there is a spontaneous formation of ketone bodies sufficient to account for the differences noted.

The observations also rule out the mechanism for the formation of acetoacetic acid suggested by Krebs and Johnson (17), whereby it was presumed acetic and pyruvic acids condense to form acetopyruvic acid, which is then decarboxylated, according to the equation

$$CH_3COOH + CH_3COCOOH \rightarrow CH_3COCH_2COCOOH \rightarrow CH_3COCH_2COOH + CO_2$$

The results also preclude any possibility by a mechanism involving either α or ω should be pointed out, however, that th liver slice experiments, may occur in the

Formation of Lower Fatty Acids—In β o theory, the assumption is made that a mol in this case hexanoic acid, is formed. If t remaining octanoic acid for the availabi involved, there should occur an accumule proceeds. The same considerations appl acetic. As a result, varying amounts of h would accumulate, depending on the relati formed. But these experiments gave no e hexanoic or butyric acid, and acetic acid, servations (10, 14), was present, if at all, with the amounts of octanoic acid catabol be offered for this phenomenon, though th experimental test: (*a*) the lower acids may oized much more rapidly than the origin acids may be so adsorbed to the surface of version to acetic acid occurs without disso fatty acids.

Nature of the 2-Carbon Intermediary—Alt fragment formed by β oxidation is not kn appears to be the most likely possibility, si pound known to form ketone bodies both (11, 13, 14). The experiments of Swend Nier (19) with carboxyl-labeled acetic acid

At first glance it is difficult to reconcile th tion of previous investigators that acetic a slower rate than the higher acids (11, 14). these *in vitro* studies the absorption of ace is slow and is the limiting factor in the ra ketone bodies. It is possible also that the acetic acid itself, but a more active derivati phosphate (20).

In view of the possible rôle of acetic a thesis of ketone bodies, suggested by th Lehninger (21) has found an enzyme in mu catalyzes the reverse reaction, the breakdow

Other Possible Breakdown Pathways of Oc acid utilized in these experiments only abou

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	Atom per cent C^{13}, excess	
	Carbonyl	Carboxyl
.....	0.84	0.83
.....	1.10	1.10
.....	0	2.20
.....	0	0

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$CH_3COCOOH + CH_3COCH_2COOH + CO_2$

The results also preclude any possibility of the formation of ketone bodies by a mechanism involving either α or ω oxidation of fatty acid (18). It should be pointed out, however, that these mechanisms, ruled out in the liver slice experiments, may occur in the intact organism.

Formation of Lower Fatty Acids—In β oxidation according to the classical theory, the assumption is made that a molecule of the next lower homologue, in this case hexanoic acid, is formed. If the latter had to compete with the remaining octanoic acid for the available active surfaces of the enzymes involved, there should occur an accumulation of hexanoic acid as oxidation proceeds. The same considerations apply to the lower acids, butyric and acetic. As a result, varying amounts of hexanoic, butyric, and acetic acids would accumulate, depending on the relative rates at which each was transformed. But these experiments gave no evidence for the presence of either hexanoic or butyric acid, and acetic acid, in agreement with previous observations (10, 11), was present, if at all, in amounts small in comparison with the amounts of octanoic acid catabolized. Several explanations may be offered for this phenomenon, though they have not as yet been put to experimental test: (*a*) the lower acids may be oxidized or otherwise metabolized much more rapidly than the original 8-carbon acid; (*b*) the fatty acids may be so adsorbed to the surface of the enzyme that complete conversion to acetic acid occurs without dissociation of the intermediate lower fatty acids.

Nature of the 2-Carbon Intermediary—Although the nature of the 2-carbon fragment formed by β oxidation is not known with certainty, acetic acid appears to be the most likely possibility, since it is the only 2-carbon compound known to form ketone bodies both *in vitro* and in intact animals (11, 13, 14). The experiments of Swendseid, Barnes, Hemingway, and Nier (19) with carboxyl-labeled acetic acid are particularly convincing.

At first glance it is difficult to reconcile this conclusion with the observation of previous investigators that acetic acid forms ketone bodies at a lower rate than the higher acids (11, 14). It is possible, however, that in these *in vitro* studies the absorption of acetic acid through the cell wall is slow and is the limiting factor in the rate of its transformation to the ketone bodies. It is possible also that the 2-carbon intermediary is not acetic acid itself, but a more active derivative, such as, for example, acetyl phosphate (20).

In view of the possible rôle of acetic acid as an intermediate in the synthesis of ketone bodies, suggested by this work, it is noteworthy that Lehninger (21) has found an enzyme in muscle mince and bacteria which catalyzes the reverse reaction, the breakdown of acetoacetic to acetic acid.

Other Possible Breakdown Pathways of Octanoic Acid—Of the octanoic acid utilized in these experiments only about one-fifth or less can be ac-

counted for by the formation of ketone bodies (Table I). The question arises, therefore, as to other possible breakdown products. One possibility is complete oxidation to CO_2. Ordinarily it is impossible to know what proportion of the respiratory CO_2 comes from the substrate and how much represents oxidation of tissue constituents. With the aid of the carbon isotope, however, the CO_2 arising from the substrate may readily be calculated. In Experiment 4, for example, the fraction of the respiratory CO_2 which would arise from complete oxidation of the octanoic acid amounts to 62 per cent (see the computation under respiratory CO_2). Of the 0.161 mM of respiratory CO_2 obtained 0.161×0.62 or 0.0998 came from the substrate. Since each mole of octanoic acid yields 8 moles of CO_2 upon complete oxidation, the 0.0998 mM of CO_2 represents the complete oxidation of $0.0998/8 = 0.0125$ mM or only $(0.0125 \times 100)/0.309 = 4.0$ per cent of the total octanoic acid which disappeared. Similar calculations for Experiments 2 and 3 give values of 4.7 and 4.0 per cent respectively. Inasmuch as a portion of the respiratory CO_2 came from spontaneous decarboxylation of the acetoacetic acid, the portion arising by oxidation is probably lower than the calculated values. It is clear that only a small amount of octanoic acid was completely oxidized.

This conclusion is borne out by consideration of the oxygen consumed, which in each experiment was very much less than would be required if extensive oxidation of the octanoic acid had occurred. The results suggest that octanoic acid may be catabolized by some as yet unknown non-oxidative reaction, or its oxidation may be coupled with a reductive process. No products containing C^{13}, other than the ketone bodies, have as yet been isolated from these experiments, but the investigation is continuing.

SUMMARY

1. When *n*-octanoic acid, labeled by the incorporation of C^{13} in the carboxyl group, was incubated *in vitro* with liver slices from fasted rats, the resultant acetoacetic acid contained the excess C^{13} equally distributed between the carbonyl and carboxyl carbon atoms. The results offer unequivocal evidence that the ketone bodies are formed by condensation of a 2-carbon intermediary resulting from β oxidation of the fatty acid.

2. Although the nature of the 2-carbon intermediary formed by β oxidation is unknown as yet, the most probable substance is acetic acid.

3. Only 20 to 25 per cent of the octanoic acid which disappeared could be accounted for either by ketone body formation or by complete oxidation to CO_2.

We express our thanks to Professor H. C. Urey for kindly supplying us with the isotopic sodium cyanide used in this study. Our thanks are due

also to the Catalytic Develop
sylvania, for its cooperation,
spectrometer for the C^{13} analy

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Some Effects of High Lipid Diets on Intestinal Elimination
IV. Saturated Fatty Acids

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In a previous investigation made in this laboratory (1) some effects on intestinal elimination were noted when diets rich in glycerides of saturated fatty acids were fed to dogs. These effects included the following: (1) constipation, when diets containing large amounts of simple triglycerides of high molecular weight (tristearin and tripalmitin) were fed; (2) diarrhea, when small amounts of simple triglycerides of acids having twelve or less carbon atoms were fed. However, the addition to the diet of as much as 20 per cent trimyristin had no effect on intestinal elimination at all. Chemical analyses showed greater than normal amounts of lipids present in the feces when the glycerides fed melted above body temperature (tristearin, tripalmitin, trimyristin, trilaurin).

Several years ago, Bosworth, Bowditch and Giblin (2) noted that bottle-fed infants receiving diets too high in fat content were subject to vomiting and diarrhea. These investigators found increased quantities of calcium soaps present in the feces of such infants and attributed the gastro-intestinal behavior of the infants to the presence of these soaps. They also claimed that the fecal lipids would be further increased in such cases by neutral fats being excluded in the soaps.

Duell, Hallman and Reifman (3), who determined the rate of absorption of fatty acids in the rat found that butyric, caproic and caprylic acids fed as sodium salts were rapidly absorbed from the intestine, while capric acid (uncombined) was absorbed more slowly. Lauric acid became solidified and remained in the stomach so that it was absorbed very slowly. Lyman (4), feeding basic diets fortified with lard, ethyl palmitate, glyceryl tripalmitate, or palmitic acid to two dogs reported that the per cent of fatty acid (actually the ether extract) in the feces after ethyl palmitate or palmitic acid was much greater than after tripalmitin or lard feedings. He attributed the increase in ether extract to undigested ethyl palmitate in the first case, but the increase in palmitic acid when that lipid was fed could not be attributed to undigested material since no digestion was required. He, therefore, assumed that free fatty acids disturb the mechanism of digestion because they are irritating to the digestive tract, causing the food mass to be discharged more slowly from the stomach, but probably increasing peristalsis and the amount of feces.

Telfur (5), studying one normal infant and three with biliary atresia and obstructive jaundice, found that when bile is completely excluded from the gut, over 70 per cent of the dried feces are composed of fatty derivatives. Further, the non-absorption and persistence of excess fatty acids in the intestine are associated with modifications of the normal calcium and phosphorus excretion. Calcium is present in the feces in excess of normal, while phosphorus, released from calcium, is absorbed and eliminated in the urine. The extent of the variation is said to depend on the concentration of free fatty acids in the intestine.

While the preceding investigators believed that a relationship existed between dietary fat, fecal lipids and, in some cases, intestinal behavior, other investigators found no relation between ingested fats and fecal fat. Krakower (6), using eight fracture patients with good elimination habits as subjects, fed normal, low fat and special diets containing added corn oil (Mazola) or added coconut oil (Nucoa) for 4-day periods. Finding no correlation between the dietary fat and the total fat content of the feces or its composition as determined by the iodine number, he concluded that fecal lipids do not represent the residual food fat when moderate or larger amounts of fat are fed. Hill and Bloor (7), basing their conclusions on observations of four cats, decided that when moderate amounts of fat are fed, the fecal fat is independent of the diet, and that fecal fat ordinarily cannot be regarded as unabsorbed fat. They found the fecal lipids to consist principally of fatty acids and their salts (soaps) with smaller amounts of cholesterol and its derivatives and a little fat. They attributed the larger amounts of fat and fatty acids present in the feces after a high fat diet to unabsorbed food fat. Holmes and Kerr (8), using human subjects, also found the fecal fat to be different from that of the diet.

Because some of these investigators had attributed variations in either the behavior of the intestines or in the lipid content of the feces to the presence of unabsorbed fatty acids or calcium salts of fatty acids, it was decided to feed some of the fatty acids, the glycerides of which had been used in our previous experiment.

PROCEDURE

Four healthy male dogs[1], the subjects in the previous experiment, were fed experimental diets. During this experiment, these dogs were kept indoors in sep-

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[1] Dog X, a thirty pound, part-spaniel; Dog C, a mixture of hound and collie, weighing about twenty-three pounds, and two fox terriers R and W, weighing seventeen and thirteen pounds, respectively.

Cap. Acid

arate cages, from which they were removed for periods of exercise. All were fed once a day and at the same time. Originally all of the dogs were given a diet of canned Pard* Dog Food, but when this article disappeared from the local markets, Dog X was fed dried food in order to conserve the scarce tinned Pard for the dogs used for the quantitative determinations. The smaller dogs, C, W, and R, were each fed the contents of a one-pound can of Pard each day. When fatty acids were to be introduced into the diet, the contents of a can of Pard (454 gm.) were thoroughly mixed with the calculated amount of acid in a mortar. The dog was then allowed to eat directly from the mortar in order to avoid loss of material through a transfer to another dish.

The large dog X, as in the previous investigation, was used principally to test the palatability of the special diets. When this dog refused to eat the food or became ill after eating it, the concentration of added fatty acid was diminished to a point where such effects were not observed before the diet was fed to the remainder of the dogs. If the dogs did not eat all of the special diets, the food was removed from the cage after half an hour. The dogs were not fed again till the next regular feeding time, 24 hours later.

When the special diets were fed, information about the intestinal behavior of the animal was obtained, as in the previous investigation, by direct observation of the animal and by chemical examination of the feces. The fecal material was collected for examination and analysed as quickly as possible after defecation in order to avoid changes in water content.

The special diets were fed for three consecutive days; the second day's ration being identified by a marker, so that the time required for the food to pass through the intestines could be easily noted.

Chemical analyses, run on the sample after mixing and grinding in a mortar until homogeneous, included moisture, total lipids, lipids soluble in neutral ether and soap fat. Iodine and acid numbers were determined on the three lipid fractions.

Moisture determinations were run simultaneously with lipid determinations in a Kaye extraction apparatus with mercury seals (9). Lipids soluble in neutral ether were extracted for twelve hours with neutral isopropyl ether, soap fats were then removed by extracting the residues for twelve hours with decinormal acid isopropyl ether. Total lipids were obtained by extracting the original samples with decinormal acid isopropyl ether for twenty-four hours and the values compared with the sum of the lipids soluble in neutral ether and soap fat for the same fractions.

All of the lipid fractions were purified by shaking at frequent intervals with petroleum ether, during a period of eight hours. The fractions were then filtered, the solvent distilled off and the residue dried in a vacuum dessicator over concentrated sulfuric acid. Iodine numbers (Wij's Method) and acid numbers were determined for the purified lipid fractions.

*Sufficient canned Pard Dog Food to complete these studies was furnished us by the Research Division of Swift and Company.

DISCUSSION AND RESULTS

Because the dogs had eaten a diet of Pard containing 20 per cent added tristearin in the previous investigation, an attempt was made to feed a diet of Pard to which 20 per cent stearic acid had been added. However, the dogs refused to eat such a diet and not until the stearic content had been reduced to five per cent would they eat their food at all. On the second day, all of the dogs were badly constipated, listless and had elevated temperatures. They refused to eat all of their rations, the amount left being inversely proportional to the size of the dog (W, 200 g.; R, 100 g.; C, 78 g.). On the third day, the symptoms of the previous day were still present, and the dogs refused to eat at all. Twenty-four hours later, the animals seemed to have recovered and ate their normal Pard diet.

Table I contains the analytical data for the fecal material in which the marker for the second day of the stearic acid diet appeared. For the purpose of comparison, data for the tristearin diet, as well as the normal diet, have been included.

Inspection of Table I shows that the addition of as little as five per cent stearic acid to the usual Pard diet of the dogs caused a reduction in the moisture content of the feces. The total lipid content of the feces of two of the dogs was less than when Pard diet alone was fed. The per cent of neutral ether extract increased in every case, and the per cent of soap fats diminished, just as was the case when the triglyceride of stearic acid was added to the diet. However, the total amount of soap present after the feeding of a diet containing 5 per cent added stearic acid was greater than after the diet with 20 per cent added tristearin.

The analytical constants of the fractions furnish little information about the composition of the lipids in the fractions. However, it can be said that the soaps isolated from the feces after feeding stearic acid do not consist primarily of stearic acid salts, since the acid numbers indicate material of much higher molecular weight.

The acid numbers ranging from 43.7 to 50.4 indicate molecular weights from 1,283 to 1,113, respectively, while the molecular weight of stearic acid is only 284. It, therefore, seems probable that, when stearic acid is fed, a great deal of the so-called soap extract in the feces does not consist of soaps of fatty acids at all.

The iodine numbers indicate a greater degree of unsaturation in the soap fraction when stearic acid was fed, than when either the Pard alone, or Pard with tristearin was fed.

Since there was no increase in calcium soaps in the feces even when stearic acid itself was added to the Pard diet, it seems reasonable to conclude that constipation resulting from a diet rich in tristearin or stearic acid is not caused by the presence of unabsorbed calcium soaps.

TABLE I

Analytical Data From Feces of Three Dogs Fed Tristearin and Stearic Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Fat by Extraction	Per Cent Lipids: Soap Fat by Diff.	Iodine Numbers: Total Lipids	Iodine Numbers: Neutral Ether Extract	Iodine Numbers: Soap Fat	Acid Numbers: Total Lipids	Acid Numbers: Neutral Ether Extract	Acid Numbers: Soap Fat
Pard	C	69.9	20.3	2.9	13.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
5% Stearic Acid		64.3	9.3	6.2	3.3	3.1	26.4	23.2	31.2	20.1	12.5	43.7
20% Tristearin		40.8	50.5	46.8	1.4	3.7	5.2	1.9	17.3	16.0	155.1	54.2
Pard	R	73.9	19.5	9.3	7.6	10.2	5.1	6.4	25.1	48.6	38.9	54.2
5% Stearic Acid		65.2	15.5	10.4	5.0	5.1	20.3	18.4	42.6	23.6	15.4	48.0
20% Tristearin		36.2	27.4	21.6	1.6	5.8	7.7	3.5	19.0	40.6	37.8	74.3
Pard	W	74.8	17.4	5.0	13.1	12.4	12.9	18.2	12.0	36.2	29.1	43.4
5% Stearic Acid		69.8	27.6	16.4	10.5	11.2	16.4	12.8	53.4	18.3	14.7	50.4
20% Tristearin		54.7	47.6	39.4	0.9	8.2	5.6	5.5	35.6	46.5	37.1	118.5

Because the dogs had all been made ill by the addition of five per cent stearic acid to the Pard diet, the experiment was not duplicated with palmitic acid. However, Dog C was fed a diet of Pard to which nine-three days all of the dogs ate a diet of Pard containing ten per cent additional lauric acid. During this period, the dogs were in good health and intestinal elimination was regular. The feces appeared normal in shape and consistency. It will be recalled that a Pard diet with 20 per cent added trilaurin in the previous

TABLE II

Analytical Data From Feces of Dog C Fed Tripalmitin and Palmitic Acid in Measured Quantities

Diet	Per Cent Lipids: Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Extract	Per Cent Lipids: Soap Fat Difference	Iodine Numbers: Total Lipids	Iodine Numbers: Neutral Ether	Iodine Numbers: Soap Extract	Acid Numbers: Total Lipids	Acid Numbers: Neutral Extract	Acid Numbers: Soap Extract
Pard	69.9	20.3	2.9	18.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
Pard +0.9% Palmitic	70.6	18.4	12.0	4.7	6.4	36.2	36.7		155.7	194.2	154.3
Pard +20% Tripalmitin	63.3	56.9	42.7	9.8	14.2	5.3	7.4	8.4	96.6	83.8	201.2

tenths of a per cent palmitic acid was added. After this diet had been fed for three days, elimination was still normal. Analytical data for the feces included in Table II are not particularly informative.

The feeding of a meal of Pard to which lauric acid had been added was not difficult to accomplish. For investigation (1) caused diarrhea with feces only semi-solid in consistency.

Table III contains the analytical data for the fecal-material eliminated after the feedings of Pard with

TABLE III

Analytical Data From Feces of Dogs Fed Trilaurin and Lauric Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Fat by Extraction	Per Cent Lipids: Soap Fat by Difference	Iodine Number: Total Lipids	Iodine Number: Neutral Ether Extract	Iodine Number: Soap Fat	Acid Number: Total Lipids	Acid Number: Neutral Ether Extract	Acid Number: Soap Fat
Pard	C	69.8	20.3	2.9	18.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
10% Lauric		48.9	12.3	7.4	5.0	4.9	15.1	12.7	16.5	18.0	15.0	42.4
20% Trilaurin		31.2	*54.6	42.0	12.6	---	---	14.4	26.8	- -	125.8	187.5
Pard	R	73.9	19.5	9.3	7.6	10.2	5.1	6.4	25.1	48.6	38.9	54.2
10% Lauric		68.8	14.6	8.4	6.3	6.2	13.9	11.2	15.4	18.4	17.4	42.9
20% Trilaurin		78.4	13.0	9.3	1.5	3.7	23.0	22.8	48.1	241.2	199.7	214.8
Pard	W	74.8	17.4	5.0	13.1	12.4	12.9	18.2	12.0	36.2	29.1	43.4
10% Lauric		72.0	13.6	7.4	6.1	6.2	13.9	12.4	17.4	18.7	19.4	46.3
20% Trilaurin		79.7	18.8	15.1	2.0	3.7	17.6	18.3	36.0	235.7	200.8	244.3

*Total Fat by Calculation.

lauric acid. Data from the trilaurin diet are added for ease in making comparisons.

Inspection of Table III reveals a slight reduction in moisture content in the case of the two terriers and a marked reduction for Dog C. This is in decided contrast to the increase in moisture content after the feeding of the triglyceride of lauric acid.

The agreement between similar lipid fractions obtained from the feces of all three dogs on the lauric acid diets should be noted. In all cases, the percentage of total lipids is less than when Pard alone was fed. The extremely low acid numbers of all the soap fractions show that lauric acid (or its salts) is not the principal component of the fecal fat when the acid is fed. An acid number of 42.4 indicates a molecular weight of 1323; lauric acid has a molecular weight of 200. This, again, is a contrast to the soap fat of the feces after feeding the glyceride of lauric acid. The higher acid numbers (187.5-244.5) indicate lower molecular weights (299-229) not too different from that of lauric acid (200). This is the best agreement between the molecular weight of the fatty acid fed and the molecular weight of the fatty acid in the material recovered from the feces of any lipid diet that we fed, excepting that of tripalmitin.

The dogs were reluctant to eat Pard containing added caprylic acid but finally consumed all of a mixture with five per cent of the acid. This diet, fed for three consecutive days, proved mildly laxative and caused semi-fluid stools.

No particular difficulty was encountered with a Pard diet containing five per cent added caproic acid for all of the dogs ate the diet with apparent relish. Following this diet, the stools were profuse, soft and pasty.

Three of the dogs could not be persuaded to eat Pard to which butyric acid had been added. Dog X, when denied other food, finally ate a mixture of Pard containing two per cent butyric acid (about 1 gm. in a pound of Pard). Following this feeding, the stools were pasty. Only part of one feeding of a mixture of Pard with four per cent butyric acid was eaten by Dog X. This feeding gave rise to pasty stools with much fluid. No chemical analysis of the stools was attempted.

The analytical data for the feces collected after diets with added caprylic and caproic acids are included in Table IV. The lipid content was much the same after the feeding of caprylic acid or its triglyceride in the concentrations used. Caproic acid, fed in the same concentration as caprylic acid, resulted in much larger quantities of fecal lipids than caprylic acid; in two cases more than the normal amount. Since the total lipids and not the soaps increased after the caproic acid feedings, it was at first suspected that the material might consist of unabsorbed caproic acid. However, the low acid numbers of the fractions indicate that free caproic acid was not present in the feces to any extent.

The low acid numbers of the soap fat present in the feces after the diets with caproic or caprylic acids indicate once more that calcium soaps are not formed in appreciable amounts, after large amounts of free fatty acids are fed.

The acid numbers of all of the soaps obtained from the feces of the three dogs after feedings with acids are in remarkable agreement. The only exception is the case of palmitic acid where less than one per cent was fed. If this acid is omitted from the list, the highest acid number of the soap fats is 50.4, obtained when stearic acid was fed to the smallest dog; the lowest value is 12.3, obtained when caprylic acid was fed to the largest dog. The average value for all dogs and all acids is 15.4. These acid numbers correspond

TABLE IV

Analytical Data From Feces of Dogs Fed Tricaprylin, Tricaproin, Caprylic Acid and Caproic Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Extract	Per Cent Lipids: Soap Fat by Difference	Acid Numbers: Total Lipids	Acid Numbers: Neutral Ether Extract	Acid Numbers: Soap Extract
Pard	C	69.8	20.3	2.9	18.0	17.4	52.8	49.4	59.9
5% Caprylic Acid		70.2	4.1	2.3	1.8	1.8	16.0	14.0	42.3
4% Tricaprylin		77.3	4.2	1.7*	1.1		58.6	91.7	95.4
5% Caproic Acid		70.1	25.0	18.3	6.7	6.7	17.3	14.0	42.5
2% Tricaproin		78.3	6.8	6.2	1.9	2.6	74.6	60.6	77.4
1% Tricaproin		76.3	5.1	3.6	3.0	1.5	155.4	141.9	106.6
Pard	R	73.9	19.5	9.3	7.6	10.2	48.6	38.9	54.2
5% Caprylic Acid		71.5	7.0	4.5	2.2	2.5	17.4	12.2	47.0
4% Tricaprylin		75.3	6.7**	5.7	1.0	–		91.2	108.8
5% Caproic Acid		72.8	22.4	16.9	5.4	5.5	15.2	12.0	43.0
2% Tricaproin		82.7	4.2	3.1	1.2	1.1	98.7	92.6	116.7
Pard	W	74.8	17.4	5.0	13.1	12.4	36.2	29.1	43.4
5% Caprylic Acid		73.2	2.9	2.5	0.8	0.4	18.2	20.1	49.1
4% Tricaprylin		76.8	7.8	6.4	0.7	1.4	108.3	91.4	128.5
5% Caproic Acid		71.9	13.4	9.5	3.8	3.9	14.0	11.4	47.1
2% Tricaproin		80.3	5.5**	4.7	0.8	---	---	69.6	123.4

*Mechanical Loss.
**Total Fat by Calculation

to molecular weights of 1113, 1326 and 1235, respectively, a finding which strongly indicates that the materials did not contain any appreciable amount of fatty acid soaps. Samples of the so-called soap fats have, therefore, been preserved for further chemical analyses.

SUMMARY

1. Measured amounts of fatty acids mixed with a standard dog food (Pard) were fed to dogs to determine whether intestinal elimination would be affected by the added acid in the same manner as by the addition of the corresponding triglyceride.

2. Stearic, lauric, caprylic, and caproic acids were the acids mixed with the standard dog food and fed to all the dogs. One dog received feedings containing less than one per cent added palmitic acid; another ate a single feeding of Pard with added butyric acid.

3. Information concerning the intestinal behavior of the animals was obtained in two ways: (1) direct observation of the animal; and (2) chemical examination of the feces.

4. Chemical components of the feces for which analyses were made included moisture, total lipids, neutral ether extract and soap fat. Acid numbers were determined for all lipid fractions after all feedings; iodine numbers were determined after stearic and lauric acid feedings.

5. Constipation was produced by 5 per cent added stearic acid.

6. The neutral ether extract increased for every animal after the 5 per cent stearic acid feedings but the so-called soap fat was less than the normal value in every case.

7. The addition of 10 per cent lauric acid to the normal diet did not affect the normal rate of elimination of the dogs.

8. The feeding of diets containing caprylic, caproic or butyric acid produced laxative effects in all cases. The intensity of the action varied inversely with the number of carbon atoms just as in the case of the triglycerides of the corresponding acids. The moisture content of the feces did not vary greatly from the normal values when caprylic or caproic acid was added to the diet. No quantitative estimations were made after feeding butyric acid.

9. The acid numbers of all of the fatty acids from the soap extracts, with the exception of the one palmitic acid experiment, ranged from 42.3 to 50.4, with an average value of 45.4. These values correspond to molecular weights of 1326, 1113 and 1236, respectively.

CONCLUSIONS

The effect of a high fat diet on intestinal elimination depends at least partially on the component acids of the fats fed. Dogs fed pure saturated fatty acids behaved in approximately the same manner as when fed the corresponding pure triglycerides. Diarrhea resulted from the feeding of diets containing caprylic, caproic or butyric acids, or the glycerides of these acids. Constipation followed feedings of stearic acid or tristearin. Although trilaurin produced diarrhea, elimination was normal after feeding lauric acid.

These effects cannot be attributed to the presence in the feces of large amounts of free fatty acids or of soaps of the fatty acids, because the low acid numbers of the total lipid and neutral ether extracts exclude the possibility of large amounts of free acid. The low acid numbers of the soap fractions rule out the possibility of large amounts of soaps of fatty acids.

The low acid numbers of all of the fractions indicate that neither the fatty acid as fed nor its soaps are present in the feces to any extent. These low acid numbers can only be explained by assuming that small amounts of free fatty acids are mixed with large amounts of non-titratable material or by a very large molecule having an acid radicle.

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Enrichment of depot fat with odd and even numbered medium chain fatty acids[1]

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ZURIER, ROBERT B., ROBERT G. CAMPBELL, SAMI A. HASHIM, AND THEODORE B. VAN ITALLIE. *Enrichment of depot fat with odd and even numbered medium chain fatty acids.* Am. J. Physiol. 212(2): 291–294. 1967.—Medium chain fatty acids shorter than laurate normally are absent in adipose tissue lipids. When these acids are present in dietary fats, they are transported via portal vein to liver where they are extensively metabolized. Transport of medium chain fatty acids into systemic circulation was achieved in rats with portacaval shunts. Enrichment of adipose tissue with medium chain fatty acids was effected by altering route of their transport to circumvent liver. Control animals and those with anastomoses were fed diets containing medium chain triglycerides of which the fatty acid constituents were C9 or C8 and C10 for periods up to 3 months. Fatty acid patterns by gas liquid chromatography were determined from samples of omental adipose tissue prior to and following portacaval shunting. Striking increases in proportions of C9 or C8 and C10 fatty acids of adipose tissue were noted in animals with portacaval shunts. By enriching depot fat with odd chain fatty acids such as pelargonate, it is possible to store appreciable quantities of fatty acids of which the terminal three carbon units are potentially glucogenic.

adipose tissue (omentum); portacaval anastomosis; pelargonate, caprylate; fatty acid transport

PLASMA FREE FATTY ACIDS (FFA) represent the transport form of lipid originating from adipose tissue and are readily available for oxidation or incorporation into lipid ester moieties of lipoprotein. Information concerning the transport and fate of FFA has been derived largely from studies of the behavior of long chain fatty acids. In contrast, medium chain fatty acids which differ markedly from the long chain fatty acids with respect to solubility and interaction with proteins usually are not constituents of the FFA transport system in the intact animal.

Alteration in the long chain fatty acid content of rat adipose tissue has been induced experimentally (7, 19, 20, 25, 28). Since ingested medium chain fats are transported as FFA in portal venous blood (14, 15, 22) directly to the liver where they are oxidized nearly completely (21), it is difficult to enrich the fat stores with medium chain fatty acids by feeding medium chain triglyceride. In the present study, an alteration of the route of transport of medium chain fatty acids to circumvent the liver was achieved by feeding odd or even numbered medium chain triglycerides to rats with portacaval shunts. In this way, medium chain FFA were transported directly into the systemic circulation. Thus, it was possible to induce striking increases in proportions of medium chain fatty acids stored in adipose tissue.

METHODS

Twenty-six male Sprague-Dawley rats weighing 250–300 g had been maintained on a nutritionally adequate stock diet deriving 26% of calories from fat (soy bean and corn oils: 50% linoleate), 54% from carbohydrate (wheat and corn meals), and 20% from protein (casein). The animals were separated into four groups depending on the sequence of portacaval anastomoses and medium chain triglyceride feeding. In *group 1* (10 rats), an end-to-side portacaval anastomosis was performed on each animal. At operation a sample of omental fat was obtained. In a control group of rats of comparable weight (*group 2;* 8 rats) a sham operation was performed at which time omental fat also was obtained. Both groups of rats were placed in individual cages, allowed to convalesce, and were fed an experimental regimen consisting of a basic fat-free diet (Nutritional Biochemical Corp., Cleveland, Ohio), supplemented with an odd numbered medium chain (C9) triglyceride (tripelargonin). The diet, as modified, was adequate in vitamins and minerals and derived 17% of calories from protein (casein), 53% from carbohydrate (sucrose), and 30% from fat (tripelargonin).

The animals in *groups 3* and *4* also were subjected to an initial sham operation, at which time samples of omen-

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TABLE 1. *Fatty acid patterns (percent of total) of adipose tissue of four groups of rats prior to and during feeding of tripelargonin and even numbered medium chain triglyceride.*

Fatty Acid	All Groups (26)	Group 1 (10)	Group 2 (8)	Group 3 (2)		Group 4 (6)	
	Prior to feeding of exptl. diets	1 Month after portacaval anastomoses and tripelargonin	1 Month after sham operation and tripelargonin	1 Month after sham operation and tripel argonin	1 Month after portacaval anastomoses and 2 months after tripelargonin	1 Month after sham operation and even numbered medium chain triglyceride	1 Month after portacaval anastomoses and 3 months after even numbered medium chain triglyceride
C6:0							
C8:0						0.5±0.3	5.4±1.2
C9:0		12.4±3.9	1.9±1.4	3.9	15.8		
C10:0				0.2	0.2	1.4±1.0	4.6±1.5
C12:0				0.2	0.3	0.6±0.5	1.1±0.2
C14:0	1.7±0.2	1.4±0.6	1.7±0.6	1.3	2.5	3.1±0.6	2.4±0.6
C16:0	25.1±4.0	28.0±5.7	29.7±5.8	31.0	28.9	32.6±1.7	28.5±5.3
C16:1	8.1±3.1	11.1±4.7	10.3±2.8	7.6	15.8	12.3±1.8	13.4±1.2
C18:0	3.1±0.8	3.1±0.8	2.7±0.4	3.5	2.7	5.6±1.7	2.6±0.4
C18:1	30.7±4.2	29.5±4.3	36.9±6.1	38.4	26.2	38.1±3.3	37.0±4.3
C18:2	28.6±5.2	11.9±7.3	17.0±12.1	12.3	2.6	4.8±2.0	5.0±2.0
Other	1.3±0.8	1.2±0.9	0.8±0.8				

Data are means ± SD; numbers of observations are noted in parentheses.

tal fat were obtained. The animals in *group 3* (2 rats) received the same experimental diet (tripelargonin-supplemented) as *groups 1* and *2*, but after 29 days on the diet they underwent portacaval anastomoses and omental biopsies. Then, they continued on the same diet for an additional 29 days, following which samples of omental fat were obtained again. The animals in *group 4* (6 rats) were subjected to the same operative procedures and in the same sequence as those in *group 3*. The rats in *group 4* consumed an experimental diet similar in all respects to those fed the other groups except that the fat source was even numbered medium chain triglyceride isocalorically substituted for tripelargonin. Thus, each animal in *groups 3* and *4* served as its own control. Animals in all groups remained healthy and gained weight during the feeding of the experimental diets. The gains in body weight of animals that had undergone sham operations were comparable to those of the animals which had received portacaval anastomoses and these animals were included in the experimental groups. Fatty acid patterns of all the omental fat samples were determined by gas liquid chromatography following transmethylation of the extracted lipid with sodium methoxide.

RESULTS

The fatty acid pattern of adipose tissue of the four groups of rats is shown in the second column of Table 1. It is apparent that the adipose tissue of all groups of rats prior to feeding of the experimental diets contained no fatty acid shorter than myristate (C14:0). Linoleate (C18:2) accounted for an appreciable proportion of the long chain fatty acids in the fat depot of the rats, reflecting their previous intake of a linoleate-rich diet. In *group 1* rats in whom tripelargonin feeding was begun following portacaval shunting, the pelargonate content 1 month after portacaval anastomosis was 12.4% of the fatty acids of the adipose tissue. In contrast, the group of sham-operated rats (*group 2*) had less than 2% pelargonate in their adipose tissue. In animals of *groups 3* and *4* that were fed odd and even numbered medium chain triglyceride, respectively, prior to and following portacaval anastomoses, the adipose tissue was enriched slightly with medium chain fatty acids prior to the shunting operation, and strikingly after operation.

The change in the long chain fatty acid patterns of adipose tissue in response to feeding of the experimental diets in the four groups of animals also is shown in Table 1. It is apparent that the feeding for 1 month of these regimens to sham-operated animals was associated with a drop in the proportion of linoleate and a concurrent rise in oleate of adipose tissue lipid. The enrichment of the adipose tissue with odd and even numbered fatty acids after feeding the corresponding triglycerides to animals with anastomoses occurred principally at the expense of linoleate in *groups 1*, *2*, and *3*. There was no appreciable change in the depot fat proportions of linoleate and oleate in animals of *group 4* during even numbered medium chain triglyceride feeding following portacaval anastomosis. However, slight decreases in the saturated fatty acid series were noted during enrichment of adipose tissue with C8:0 and C10:0 fatty acids.

DISCUSSION

It has been possible to enrich adipose tissue glycerides with appreciable amounts of odd and even numbered medium chain fatty acids by shunting the portal venous blood of rats fed the parent triglycerides into the systemic circulation. Thus, if they are made to bypass the liver, medium chain fatty acids, though they are presumably unesterified, readily enter adipose tissue. Also, in this way, the adipose tissue can be enriched with odd num-

d fatty acids of which the terminal three carbon units are potentially glucogenic.

In contrast, studies of the distribution of albumin-bound ^{14}C-labeled palmitate in rats 15 min after intravenous injection have disclosed a general uptake of the label by liver, muscle, and other organs. It is of particular note that adipose tissue had no detectable radioactivity (6). This was the case in the fed as well as the postabsorptive animal. On the other hand, injected chylomicrons were taken up primarily by adipose tissue in the fed animal. It appears that re-esterification of long chain free fatty acids and their incorporation into lipoprotein may be necessary before such fatty acids can be stored in adipose tissue (5, 8, 10).

The fatty acids derived from orally administered medium chain triglyceride are transported via the portal vein in the unesterified form (14, 15, 22), while those derived from long chain triglycerides are transported in lymph as lipid ester moieties of chylomicrons (4, 17). The medium chain fatty acids are extensively oxidized by the liver and feeding medium chain fats does not lead to any appreciable deposition in adipose tissue lipids of the intact animal (11, 24, 27). In the present study, the small amounts of medium chain fatty acids found in the adipose tissue of the sham-operated animals may be related to a slight degree of chylous transport of medium chain acids in triglyceride form (23). There was more noate (10:0) than caprylate (C8:0) in the sham-rated rats in group 4 despite the fact that decanoate was only 20% of the triglyceride that was fed. This suggests a greater degree of chylous transport for decanoate than for caprylate. Also, more decanoate than caprylate may have been transported from the liver to the adipose tissue as a constituent of plasma lipoprotein.

Medium chain triglycerides are hydrolyzed in the intestine faster than long chain triglycerides (3, 9, 13) and are absorbed under circumstances which adversely affect long chain glyceride absorption (16, 18, 29). This may be due to the fact that the products of hydrolysis of medium chain triglycerides are more soluble than those derived from long chain triglycerides (7), permitting absorption directly through the gut mucosa and into the portal capillary bed without re-esterification. The same mechanism may apply for entry of medium chain fatty acids into the adipose tissue cell. Indeed, adipose tissue displays cellular processes characteristic of pinocytosis (21), and it has been suggested that pinocytosis may be involved in the absorption of fat by the intestine (26).

Another mechanism may be considered. As chain lengths of fatty acids decrease, the fatty acid-albumin bond is weakened (1, 12). It is conceivable that when a medium chain FFA, such as pelargonate, comes in contact with the membrane of the adipose tissue cell, this bond is easily broken, facilitating entry of the fatty acid to the cell. In any event, the greater affinity which serum albumin has for long chain fatty acids than for medium chain fatty acids is a pronounced physicochemical difference which, in part, may help to account for the different behavior of these fatty acids in biological systems.

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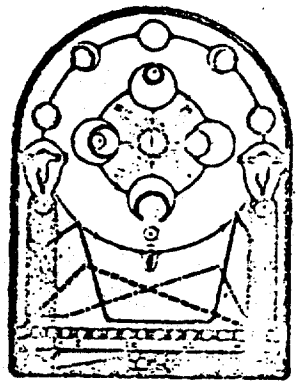

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Nutritional Evaluation of Medium-Chain Triglycerides in the Rat

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S 1

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Abstract

Nutritional evaluation of a medium-chain triglyceride (MCT) preparation, containing about 75% octanoic acid and 25% decanoic acid, was carried out in short- and long-term experiments in rats. A casein diet containing 19.6% MCT and 2.5% safflower oil, the latter to supply essential fatty acids, was compared with similar diets containing conventional dietary fats. Data obtained in a 47-week study showed that the MCT diet supported normal growth and development. At autopsy, carcass protein and ash levels, vital organ weights and composition were similar to those in rats fed conventional fats. Histological study showed that intestinal and liver sections were normal after 47 weeks on the MCT-containing diet. In general, rats fed MCT had slightly lower growth rates and caloric efficiency values, less carcass fat and smaller epididymal fat pads than animals fed conventional dietary fats. Little C_8 and C_{10} were found in depot fat. The MCT diet also supported normal reproducton, as indicated by litter size and number. During lactation the volume of milk secreted by the rats receiving the MCT diet was smaller and contained a lower level of fat than that secreted by the rats receiving an oleo oil diet, resulting in slower gains in weight in the MCT group. After weaning, growth of the rats fed MCT compared favorably with that attained by the animals on the diet containing oleo oil.

21% 2.5%

Introduction

In recent years a number of papers have appeared which indicate that the medium-chain triglycerides (MCT) are of particular value in the nutritional management of individuals who are unable to effectively absorb dietary fat. MCT is of special importance in malabsorption syndromes which arise from a deficiency of pancreatic lipase (1), bile salts (2), or intestinal absorptive area (3). Because of ease of absorption (4–8), the calories in MCT are available to maintain positive caloric balance and body weight in individuals who are unable effectively to absorb conventional dietary fats.

Only a few papers have appeared which demonstrate the nutritional value of the medium-chain triglycerides. Kaunitz et al. (9) demonstrated that male rats which were fed diets containing MCT for a year weighed significantly less than animals fed a lard diet and had higher caloric requirements to maintain weight than animals fed a lard or a fat-free diet. Kaunitz et al. also concluded that linoleic acid requirements are less with MCT than with long-chain triglycerides (C_{12-18}) (10). In the past few years the authors' laboratory has been engaged in a series of studies designed to obtain more information on the nutritional and biochemical aspects of MCT so that this unique triglyceride might be used for special dietary needs. Although much of this work has not yet been published, two long-term studies with MCT, a 47-week growth study beginning with weanling rats and a reproduction and lactation study, are now reported.

Methods

In the long-term growth study, groups of 15 male and 15 female rats of the Wistar strain were fed diets which differed only in the source of dietary fat. Throughout the 47-week study the animals were housed in individual screen-bottom cages in an air-conditioned animal room. The diets contained sufficient fat to supply 40% of the calories from MCT, oleo oil, butter fat, coconut oil, corn oil, and safflower oil. Safflower oil (2.5%) was added to the diets containing MCT, oleo oil, butter fat, and coconut oil to insure adequacy of the essential fatty acids in all diets. The diets also supplied 38% of the calories from carbohydrate, 22% of the calories from protein, and adequate amounts of vitamins and minerals (Table I).

Records of food intake were kept for the calculation of caloric efficiency and net absorption of fat, protein, and calcium. At intervals during the study, feces were collected daily, pooled in weekly samples, and analyzed for fat (11), total nitrogen (Kjeldahl method), and for calcium (12). At specified intervals, blood was obtained from the tail vein and analyzed for cholesterol by method of Abell et al. (13).

At the termination of these studies the rats were killed, and the weights of the livers, kidneys, spleen, heart, adrenals, femurs, testes, and epididymal fat pads were determined. Liver and intestine were examined histologically; liver and carcass were analyzed for fat by method of Folch et al. (14) and protein by Kjeldahl. Phospholipid levels in the liver were also determined (15). The fat content of the fat pads was determined (11), and fatty acids were measured by gas chromatography (16) after methylation (17).

In the reproduction and lactation study, groups of young adult male and female rats of the McCollum-Wisconsin Strain were fed the MCT and oleo oil diets, as above, and a third group was given a low-fat diet containing 2.5% safflower oil (Table I, Diet 7). After three weeks on these diets the animals were mated. The females were housed in a separate room, and each

TABLE I
Composition of Diets

	Diet 1–6 40% Fat calories	Diet 7 Low fat
	%	%
Fat[a]	21.0	2.5
Casein (ANRC 91.4% protein)	26.2	26.2
Amidex[b]	44.5	63.0
Nonnutritive fiber	4.0	4.0
Mineral mixture[c]	4.0	4.0
Vitamin mixture[d]	0.35	0.35

[a] Diets 1–4 contained mainly MCT, oleo oil, butterfat, and coconut oil respectively, with 2.5% safflower oil added to insure adequate essential fatty acids. (The level of the fat in the MCT diet was increased slightly since MCT provides only 8.3 cal/g.) Diets 5 and 6 contained corn oil and safflower oil respectively.

[b] Partially hydrolyzed corn starch, Corn Products Company, New York.

[c] J. H. Jones and C. Foster (J. Nutr. 27, 245, 1942) with 10 ppm F added as NaF.

[d] H. P. Sarett and La P. Skipper (J. Nutr. 52, 525, 1954). Ascorbic acid omitted. In addition, 0.015 g Oleum Percomorphum and 0.005 g dl-α tocopherol acetate were added per 100 g diet.

TABLE II

Weight Gains and Caloric Efficiency Values for Male and Female Rats Fed Different Dietary Fats for 47 Weeks

Dietary fat	Males				Females			
	4-Week caloric efficiency	Weight gain after 4 wks	Weight gain after 8 wks	Weight gain after 47 wks	4-Week caloric efficiency	Weight gain after 4 wks	Weight gain after 8 wks	Weight gain after 47 wks
	g gain/1,000 cal.	g	g	g	g gain/1,000 cal.	g	g	g
MCT[a]	115 ± 10	181 ± 21	332 ± 44	637 ± 94	101 ± 5	139 ± 12	209 ± 21	375 ± 83
Oleo oil[a]	119 ± 6	200 ± 22[b]	354 ± 39	668 ± 115	99 ± 5	137 ± 10	208 ± 20	403 ± 76
Butter fat[a]	118 ± 6	201 ± 15[b]	356 ± 32	669 ± 97	101 ± 8	146 ± 16	223 ± 29	417 ± 81
Coconut oil[a]	121 ± 5	197 ± 14[b]	353 ± 25	715 ± 102[b]	98 ± 16	142 ± 13	215 ± 18	432 ± 76
Corn oil	122 ± 9	196 ± 19[b]	353 ± 38	630 ± 62	100 ± 5	140 ± 11	220 ± 19	414 ± 39
Safflower oil	119 ± 7	188 ± 17	345 ± 37	674 ± 71	103 ± 6	139 ± 15	212 ± 21	400 ± 61

[a] Plus 2.5% safflower oil.
[b] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE III

Weights of Epididymal Fat Pads and Carcass Composition of Rats Fed Different Dietary Fats for 47 Weeks

Dietary fat	Epididymal fat pads		Carcass Composition[b]			
			Weight	Fat	Protein	Ash
	g	% body wt.	g	%	%	%
MCT[a]	14.6	2.2	580 ± 80	22.1 ± 5.9	19.4 ± 1.6	3.0 ± 0.3
Oleo oil[a]	18.7	2.6	607 ± 91	22.4 ± 4.9	20.1 ± 2.0	3.1 ± 0.5
Butter fat[a]	19.1	2.7	612 ± 83	24.7 ± 5.5	19.4 ± 1.9	3.0 ± 0.6
Coconut oil[a]	23.2[c]	3.1[c]	644 ± 89[c]	26.7 ± 6.1[c]	18.2 ± 1.7	2.8 ± 0.5
Corn oil	16.7	2.5	572 ± 51	23.7 ± 3.3	19.0 ± 0.8	3.1 ± 0.4
Safflower oil	17.9	2.5	610 ± 61	22.6 ± 4.0	19.8 ± 1.1	3.0 ± 0.3

[a] Plus 2.5% safflower oil.
[b] Does not include liver, heart, epididymal fat pads, or gastrointestinal tract.
[c] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE IV

Fatty Acid Content of Dietary and Epididymal Fat in Rats Fed Various Dietary Fats for 47 Weeks

	Fatty acids, %											
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:4}$	Other
Dietary Fat												
MCT[a]	51.0	35.0	2.0		0.9			1.4	9.0			0.7
Oleo oil[a]				2.9	22.1	4.8	13.4	43.2	12.5			1.1
Butter fat[a]	1.9	3.3	2.9	8.1	22.8	3.8	10.5	23.3	13.3			10.1
Coconut oil[a]	8.1	7.2	36.8	17.2	10.0		2.4	7.2	11.0			0.1
Corn oil					13.4		1.4	26.2	57.8			1.2
Safflower oil					6.7		1.9	10.0	80.8	0.2		0.4
Epididymal Fat												
MCT[a]	0.4	4.0	1.6	2.2	21.9	8.3	2.3	30.8	25.1	0.7	0.4	1.5
Oleo oil[a]				1.4	16.2	4.0	5.0	59.8	10.6	1.0	0.2	1.8
Butter fat[a]			1.1	5.1	22.4	6.1	4.2	45.2	11.7	1.9	0.1	2.2
Coconut oil[a]		0.7	20.3	12.9	15.7	4.6	1.1	26.2	17.4	0.4	0.1	0.6
Corn oil				0.3	11.9	1.2	1.6	33.0	50.2	0.6	0.6	0.6
Safflower oil				0.4	9.4	1.0	1.8	17.4	68.0	0.4	0.9	0.7

[a] Plus 2.5% safflower oil.

was kept in a large plastic cage with wood shavings during pregnancy and lactation. After weaning, selected young from each litter were housed in the screen-bottom cages and raised to maturity for further reproduction and lactation studies in succeeding generations.

In order to estimate the volume of milk secreted during lactation, the mother was removed from her litter for a six-hour period every third day. Milk secretion was estimated by the increase in weight of the pups measured just prior to and immediately after a one-hour nursing period after the return of the mother. In a separate reproduction study, milk was obtained from the lactating rats receiving the MCT and oleo oil diets, by the method of Cox and Mueller (18), for proximate analysis and determination of fatty acid content.

Growth Studies

Weight gains of the rats receiving the MCT, oleo oil, butterfat, coconut oil, corn oil, and safflower oil diets after 4, 8, and 47 weeks and caloric efficiency values for the first four weeks are shown in Table II. Weight gains with MCT were only slightly less than with other fats. After four weeks the male rats fed the MCT diet gained 181 g compared to gains of 200 g with oleo oil, 201 g with butter fat, 197 g with coconut oil, 196 g with corn oil, and 188 g with safflower oil. Four-week caloric efficiency values were also similar with all dietary fats, varying between 115 and 122 g gained per 1,000 calories. At the end of 47 weeks, weight gains with MCT, in both males and females, were moderately lower than those with most of the other dietary fats. There was a significant difference only between the values with the MCT and coconut oil diets in the male rats.

Mortality was not markedly different in the groups receiving the various fats during the study. During the 47 weeks an average of 2.5 rats died per group of 15 males and 1.7 per group of 15 females; in the groups receiving MCT, 3 males and 2 females died during the study.

Organ weights of the liver, kidneys, spleen, heart, adrenals, femurs, and testes were determined at the end of the study and were similar in all groups; data are not given. Histological examination of the liver and intestine also show no marked differences among the groups receiving the different dietary fats.

Weights of the epididymal fat pads were found to be affected by the type of dietary fat, as shown in

Table III. In the group receiving the MCT diet, the fat pads were 2.2% of the body weight, as compared with values of 2.5 to 3.1% in the groups receiving the other dietary fats. These data correlate fairly well with the level of fat in the carcass. Animals consuming MCT had the lowest level of carcass fat. Levels of protein and ash in the carcass were similar with all dietary fats.

Fatty acid composition of depot fat was influenced by dietary fat and varied widely among the groups. In each group, epididymal fat composition was quite similar to that of carcass fat; data on fatty acids in epididymal fat are shown in Table IV along with composition of dietary fats. The high levels of C_{12} in coconut oil, and $C_{18:2}$ in corn oil and safflower oil were reflected in the high levels of these fatty acids in the epididymal fat. However low levels of C_8 and C_{10}, 0.4 and 4.9% respectively were found in the fat pads of the rats fed MCT although these fatty acids comprised about 85% of the dietary fat. High levels of palmitic acid (21.9%) and of oleic acid (30.8%) were in the fat pads of the rats fed MCT although only traces of these fatty acids were in the diet. Examination of the data suggest that fatty acids such as C_{12}, C_{14}, and $C_{18:2}$ are apparently utilized to a great extent for direct incorporation into tissue fat; C_8 and C_{10} are rapidly metabolized to smaller units, and little of these is directly incorporated into tissue fat; C_{16} and $C_{18:1}$ are the main fatty acids synthesized.

It is interesting that the level of $C_{18:2}$ in the epididymal fat of the MCT and coconut oil-fed groups were 2.8 and 1.6 times the levels in the diets respectively whereas, with the other diets, the epididymal fat contained 0.8 to 0.9 as much $C_{18:2}$ as the dietary fats. This may be related to the presence of the shorter-chain fatty acids in MCT and coconut oil; these are mostly metabolized, thereby indirectly increasing the relative amount of $C_{18:2}$ in the fat made available to the animal.

Total plasma cholesterol levels measured at intervals during the study (Table V) were lower in male rats receiving the MCT diet than in those receiving the other dietary fats. This relationship was not apparent in female rats. In both sexes the highest levels were found in the groups receiving the coconut oil diet. At the end of the study, blood samples for more complete cholesterol analyses were obtained by cardiac puncture after the animals were fasted for 18 hours. Under these conditions the lowest values were found in the animals on the corn oil and safflower oil diets; with MCT the values were higher. The differences in findings between the earlier values and the terminal values may have been attributable in part to differences in conditions under which the blood samples were obtained or may reflect changes in age of the animals or in length of time on the diets.

Total liver lipids and cholesterol levels were lower in both male and female on the MCT diet than in those receiving the other dietary fats (Table VI). Phospholipid levels were similar on all diets and were apparently not affected by the type of dietary fat in both males and females. The difference between total lipids and the sum of phospholipids and cholesterol presumably represents the triglyceride fraction. This

TABLE V

Plasma Cholesterol Levels in Rats Fed Various Dietary Fats for 47 Weeks

	Weeks							
	7	14	21	35	47			
	Plasma cholesterol							
	Total				Total	Free	Esterified	Esterified
	mg/100 ml				mg/100 ml			%
Males								
MCT[a]	84 ± 9	85 ± 13	92 ± 15	99 ± 15	100 ± 28	27 ± 7	73 ± 23	73 ± 5
Oleo oil[a]	105 ± 18[b]	110 ± 20[b]	116 ± 26[b]	117 ± 23[b]	86 ± 26	22 ± 8	64 ± 20	74 ± 5
Butter fat[a]	110 ± 16[b]	108 ± 15[b]	123 ± 19[b]	126 ± 29[b]	92 ± 28	22 ± 7	72 ± 21	76 ± 3
Coconut oil[a]	112 ± 9[b]	115 ± 12[b]	128 ± 13[b]	135 ± 12[b]	113 ± 28	25 ± 7	89 ± 27	78 ± 3[b]
Corn oil	110 ± 21[b]	104 ± 24[b]	118 ± 25[b]	115 ± 19[b]	81 ± 17	15 ± 4[b]	66 ± 13	82 ± 1[b]
Safflower oil	100 ± 17	97 ± 17[b]	109 ± 23	105 ± 18	82 ± 23	15 ± 7[b]	67 ± 18	82 ± 3[b]
Females								
MCT[a]	109 ± 13	107 ± 11	119 ± 19	126 ± 21	124 ± 26	33 ± 9	92 ± 19	74 ± 4
Oleo oil[a]	106 ± 15	101 ± 13	107 ± 13	116 ± 25	102 ± 36	26 ± 9	73 ± 27	74 ± 3
Butter fat[a]	110 ± 13	108 ± 9	125 ± 19	122 ± 23	126 ± 15	33 ± 6	92 ± 13	74 ± 4
Coconut oil[a]	124 ± 20[b]	125 ± 19[b]	142 ± 25[b]	148 ± 28[b]	125 ± 31	28 ± 8	97 ± 24	79 ± 3[b]
Corn oil	96 ± 14[b]	96 ± 11[b]	103 ± 19[b]	112 ± 17	93 ± 37[b]	17 ± 9[b]	76 ± 29	82 ± 3[b]
Safflower oil	88 ± 13[b]	83 ± 11[b]	101 ± 12[b]	107 ± 13[b]	90 ± 16[b]	17 ± 5[b]	74 ± 13[b]	82 ± 4[b]

[a] Plus 2.5% safflower oil.
[b] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE VI

Liver Lipid Composition in Rats Fed Various Dietary Fats for 47 Weeks

Dietary fat	Liver weight	Total lipid		Phospholipids	Cholesterol
Males	g	mg	%	mg/g	mg/g
MCT[a]	14.3 ± 1.9	839 ± 236	6.2 ± 1.2	26 ± 2	2.5 ± 0.4
Oleo oil[a]	14.5 ± 2.0	991 ± 380	6.7 ± 1.0	24 ± 3[b]	3.1 ± 0.4[b]
Butter fat[a]	13.9 ± 1.6	1040 ± 375	7.4 ± 2.2	24 ± 2[b]	3.0 ± 0.5[b]
Coconut oil[a]	16.1 ± 3.6	1317 ± 435[b]	7.8 ± 1.0[b]	24 ± 3[b]	2.8 ± 0.3[b]
Corn oil	13.8 ± 1.9	1073 ± 243	7.7 ± 0.9[b]	22 ± 3[b]	4.0 ± 0.6[b]
Safflower oil	15.7 ± 3.9	1529 ± 1052[b]	9.1 ± 3.6[b]	23 ± 3[b]	4.7 ± 1.3[b]
Females					
MCT[a]	9.8 ± 1.7	705 ± 282	7.1 ± 1.8	24 ± 3	3.2 ± 0.3
Oleo oil[a]	10.4 ± 1.8	937 ± 277[b]	8.9 ± 1.7[b]	24 ± 3	2.5 ± 0.3
Butter fat[a]	10.9 ± 1.8	857 ± 238	7.8 ± 1.2	24 ± 2	2.4 ± 0.2
Coconut oil[a]	11.1 ± 2.3	988 ± 266[b]	8.9 ± 1.5[b]	25 ± 2	2.4 ± 0.3
Corn oil	11.5 ± 2.0	894 ± 182[b]	7.9 ± 1.7	24 ± 3	3.8 ± 0.4[b]
Safflower oil	10.1 ± 1.8	907 ± 282	8.9 ± 1.7[b]	24 ± 2	3.1 ± 0.3[b]

[a] Plus 2.5% safflower oil.
[b] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE VII

Net Absorption of Dietary Fat, Protein, and Calcium from Diets with Different Dietary Fats

	Percentage of Dietary Intake				
	Fat absorp- 35 wks	Protein absorp- tion 35 wks	Calcium absorption		
			3 wks	10 wks	21, 35, and 47 wks (Average)
	%	%	%		
Males					
MCT[a]	97	94	70	41	20
Oleo oil[a]	78	93	68	41	16
Butter fat[a]	89	94	71	45	17
Coconut oil[a]	96	94	73	44	20
Corn oil	95	93	67	39	20
Safflower oil	95	91	74	43	21
Females					
MCT[a]	98	91	74	35	26
Oleo oil[a]	85	93	72	25	22
Butter fat[a]	94	91	74	36	17
Coconut oil[a]	96	92	78	42	21
Corn oil	97	91	72	40	15
Safflower oil	97	94	70	38	22

[a] Plus 2.5% safflower oil.
100 g of diet supplied about 21 g fat, 23.6 g protein from casein, and 454 mg of calcium (447 mg from $CaCO_3$, and 6.8 mg from casein).

fraction was also lower in the MCT groups than in those on the other diets.

Net absorption of fat, protein, and calcium during various periods of the study are shown in Table VII. The values were calculated from dietary intakes and fecal excretion with no correction for endogenous losses. Values were obtained on group-pooled samples and do not permit statistical comparisons. The net absorption of MCT was higher than that of the other dietary fats; there was little difference in protein absorption. Calcium absorption was apparently not markedly affected by the type of fat in the diet. However retention of calcium decreased with age; about 70% during the third week, 30 to 40% in the 10th week, and about 20% later in the study.

Reproduction and Lactation Study

The F_0 generation (young adult male and female rats of the McCollum-Wisconsin Strain) were maintained on the MCT diet, the oleo oil diet, and the low-fat diet for three weeks prior to mating. The average number of pups per litter and birth weights of the F_1 generation are shown in the upper half of Table VIII. Findings were similar on all three diets. Weight gains during weaning were lower on the low-fat diet than on the MCT or oleo oil diet; at 21 days male rats on the MCT diet averaged 45 g and those on the oleo oil diet 47 g whereas those on the low fat diet weighed only 39 g. Mortality during the lactation period was 6, 7, and 2% on the three diets respectively.

After weaning the F_1 rats were raised on the same diets fed to their mothers. Weights at 49 and 105 days of age were again lowest in the group receiving the low-fat diet. This difference in weight gain is related in part to food intake since caloric efficiencies were similar on all three diets.

At 12 weeks of age each group of F_1 rats was divided into three subgroups; one subgroup was continued on the same diet whereas the two other subgroups were switched to the diets containing one of the other two fats, thus providing nine experimental groups, as shown in the lower portion of Table VIII. After three weeks the F_1 females were mated; data on their pups, the F_2 generation, are summarized in Table VIII. The number of pups per litter and birth weights were also similar for all the subgroups. However weight gains of the pups during lactation were related to the fat in the diet of the mothers during this period. In general, the highest weights at weaning (21 days) were found in the groups on the oleo oil diet except for the slightly low value in that group which had previously received the low-fat diet. Intermediate weaning weights were found in the groups receiving the MCT diet, and the lowest weaning weights were found in the groups receiving the low-fat diet.

Mortality was relatively high in two of the groups receiving MCT, the one previously on MCT (22%) and that previously on the low-fat diet (20%), but not in the group previously on the oleo oil diet (6%). Mortality was 7% or less in the other six subgroups. Subsequent growth of these animals showed no marked differences, as shown by weights of males at 63 days. Determination of the amount of milk secreted by the mothers of each subgroup suggested that this factor may have affected weight gain and mortality.

The increase in weight of the litters during a timed lactation period was used as an index of milk secretion; these values are summarized in Table IX. During these seven periods the F_0 mothers secreted on the average about 40, 41, and 43 g of milk on the MCT, oleo oil, and low-fat diets respectively, showing no difference because of diet. There was much greater variation in milk secretion by the F_1 mothers. However milk secretion was quite low in those animals which had received the MCT diet for two generations.

The composition of the milk obtained from F_0 mothers receiving the MCT and oleo oil diets was determined in a previous study. Data in Table X suggest that the level of fat in the milk of the animals receiving MCT is slightly lower than that in the milk of the rats receiving oleo oil. Although 85% of the

TABLE VIII

Birth Weight and Body Weights of Rats Born and Nursed by Mothers Receiving MCT, Oleo Oil, and Low-Fat Diets

Dietary fat[a]	Pups per litter	Day: Birth	6	12	18	Day: 21	49	63	105
		Male and Female				Male			
		Weight, g per rat				Weight, g per rat			
F_1 Generation									
MCT	9.0	6.4	13	21	34	45	181		309
Oleo oil	9.1	6.1	14	24	34	47	186		326
Low fat	9.6	6.4	13	22	29	39	165		286
F_2 Generation									
MCT	9.2	6.5	12	23	35	45		261	
Oleo oil } MCT	7.0	6.7	12	23	36	45		242	
Low fat } MCT	9.4	6.6	12	23	36	43		243	
MCT	9.4	6.8	13	25	39	49		249	
Oleo oil } Oleo oil	9.2	6.3	12	24	39	47		244	
Low fat } Oleo oil	10.5	6.0	11	23	35	43		248	
MCT	10.8	6.2	11	21	31	36		241	
Oleo oil } Low fat	8.8	6.4	12	25	36	39		245	
Low fat } Low fat	9.3	6.5	12	23	32	38		243	

[a] All diets contained 2.5% safflower oil.

TABLE IX

Milk Secreted by F_0 and F_1 Generation Lactating Rats Receiving MCT, Oleo Oil, and Low-Fat Diets[a]

Dietary fat[b]	Day 3	6	9	12	15	18	21	Total
	g milk							
F_0 Generation								
MCT	3.3	4.7	5.0	5.5	7.1	7.3	7.3	40.2
Oleo oil	4.3	6.5	4.5	5.3	6.5	6.0	6.0	41.2
Low fat	4.5	5.6	6.3	6.2	5.4	6.7	8.4	43.1
F_1 Generation								
MCT } MCT	1.7	0.8	4.0	2.3	4.0	5.2	6.3	24.3
Oleo oil } MCT	1.3	2.0	2.8	4.3	6.2	9.0	8.8	34.4
Low fat } MCT	2.4	3.9	5.0	5.0	6.7	8.7	5.5	37.2
MCT } Oleo oil	2.2	3.6	6.2	7.0	6.6	10.2	9.2	45.0
Oleo oil } Oleo oil	1.5	2.2	4.8	5.8	6.3	10.8	9.8	41.2
Low fat } Oleo oil	2.2	4.8	7.5	8.7	8.3	8.3	10.0	49.8
MCT } Low fat	1.8	6.5	5.8	4.0	7.5	11.2	10.0	46.3
Oleo oil } Low fat	2.0	4.2	5.2	4.4	7.6	9.6	8.0	41.0
Low fat } Low fat	1.6	3.9	4.4	5.3	6.6	7.7	9.1	38.6

[a] Milk secretion was estimated as the increase in weight of each litter during a one-hour lactation period; the mother was removed from the litter for six hours beforehand.
[b] All diets contained 2.5% safflower oil.

TABLE X

Fatty Acid Composition of Milk Obtained from Lactating Rats Receiving MCT- or Oleo Oil-Containing Diets

Dietary fat[b]	Milk fat	Fatty acid composition of milk fat[a] — Fatty acid No. 8	10	12	14	16	18	16:1	18:1	18:2	18:3	20:4	Other
	%												
MCT	8.2	6.5	16.8	10.3	11.5	29.9	4.7	0.9	11.7	6.5	0.3	0.8	0.4
Oleo oil	9.8	2.2	5.8	4.4	6.6	20.8	9.4	2.4	36.7	8.0	0.9	0.4	2.8

[a] Milk was obtained on 10th day of lactation.
[b] Diets also contained 2.5% safflower oil.

dietary fatty acids were C_8 and C_{10} in the MCT group, these constituted only 24% of the milk fat fatty acids. In contrast, the fatty acids in the milk secreted by the oleo oil group were similar to those contained in the dietary fat. Fatty acid composition of these milks show appreciably higher levels of saturated fatty acids C_8 to C_{16} in the milk of the MCT group and markedly more $C_{16:1}$, C_{18}, and $C_{18:1}$ in the milk of the rats fed oleo oil. It is apparent that the rats fed MCT were required to synthesize a large portion of the fatty acids secreted in the milk fat.

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Improved Technique for Analysis of Free Fatty Acids in Butteroil and Provolone Cheese

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Abstract

A method is described for isolating the free fatty acids (FFA) of butteroil and Provolone cheese, using the silicic acid–KOH column of McCarthy and Duthie. The FFA were eluted from the column with phosphoric acid in diethyl ether. After addition of methanol, the FFA and excess phosphoric acid were titrated with methanolic KOH. The potassium phosphate salts were sedimented and the supernatant evaporated to dryness under nitrogen. Experiments indicated no saponification of triglycerides and no esterification of FFA during the above manipulations. Butyl esters were prepared using sulfuric acid as a catalyst, and esters from butyrate through linolenate were estimated with gas-liquid chromatography, using heptanoic and heptadecanoic acids as internal standards. FFA were determined in three different samples of butteroil and in Provolone cheese. In butteroil the short-chain FFA were low and the long-chain FFA were high in concentration. In Provolone cheese the short-chain FFA were extremely high. Butyric acid was highest in concentration of all the FFA.

Free fatty acids (FFA) are known to contribute to desirable and undesirable flavor in foods. This is especially true in cheese and other dairy products containing the volatile short-chain fatty acids. Day and co-workers (3) have modified the ion exchange technique of Hornstein et al. (6) to analyze the FFA in milk fat, and have coupled it with the column techniques of Wiseman and Irvin (13) and Keeney (7) to analyze the total spectrum of FFA in Cheddar (2), Blue (1), and Swiss (8) cheeses. These workers found significant quantities of short- and long-chain FFA in these foods.

The present paper describes an improved technique for isolating and quantifying the FFA from butyrate through linolenate as they occur in butteroil and Provolone cheese. The technique is a modification and extension of the work of McCarthy and Duthie (10) and offers distinct advantages over existing methods.

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Materials and Methods

Reagents. All solvents except Skellysolve B were analytical reagent grade and most were distilled prior to use. Merck methanol, Mallinkrodt isopropanol, and Skellysolve B were distilled over potassium hydroxide. Mallinkrodt absolute diethyl ether was distilled over calcium hydride. Fisher n-butanol was used without distillation. Mallinkrodt reagent grade 85% phosphoric acid was untreated. Chromatographically pure standard fatty acids were obtained from Applied Science Laboratories, College Park, Pennsylvania, and the Hormel Foundation, Austin, Minnesota. Butyric and heptanoic acids were purchased from Eastman Organic Chemicals, Rochester, New York, and were white-label grade.

Fifty grams of Mallinkrodt 100-mesh silicic acid lot no. 2847 was washed three times, each time with 150 ml ether containing phosphoric acid (2.5% v/v). This was followed by two washes of 150 ml each of ether. The dried silicic acid was then washed exhaustively with distilled water, redried, and activated at 175 C for 18 hr.

Isolation of the FFA from butteroil. Butteroil was prepared by melting butter at 40 C and centrifuging to separate the serum. The butteroil was then decanted and filtered at about 40 C through anhydrous sodium sulfate.

Five to 10 mg of 7:0 and 17:0 acids were weighed, dissolved in a small amount of Skellysolve B, and added to 25 to 30 g of butteroil. The butteroil was dissolved in 150 ml of Skellysolve B and added to a 4-g silicic acid column prepared as described by McCarthy and Duthie (10). The column was 1.5 by 40 cm and fitted with a sintered glass disc and a Teflon stopcock. Slight air pressure was applied to the column to obtain a flow rate of approximately 23 ml/minutes. The column was washed with 75 ml ether to remove the lipids and the FFA were eluted with 60 ml of ether containing concentrated phosphoric acid (2.5% v/v) at a flow rate of 10 ml/minute. The column was then washed twice with 40-ml portions of ether. An

equal volume of methanol was added to the combined eluates and the solution titrated to the phenolphthalein end point with 1 N methanolic KOH under nitrogen. The potassium phosphate salts were removed by centrifugation and the clear supernatant evaporated at 50 C on a rotary evaporator to about 5 ml. This was transferred to a 16 by 125 mm screw-cap round-bottomed test tube and evaporated to dryness at 50 C, using a stream of nitrogen. The sample was then ready for esterification.

Isolation of the FFA from Provolone cheese. Provolone cheese, aged about six months, was obtained from the University of Wisconsin dairy plant. A second sample of Provolone cheese, extra piccante type, was obtained commercially. Prior to extraction, the cheese was diced and frozen at −20 C. The frozen cheese was then pulverized to a powder in a Waring Blendor. Five grams of cheese was acidified to pH 2, as determined by acidifying a separate sample by addition of 50% sulfuric acid. Five to 10 mg each of 7:0 and 17:0 were added in a small amount of Skellysolve B to the cheese with thorough mixing. Nine grams of silicic acid were added to the cheese, mixed well, and this material applied as a cap to the column, as described by Bills and Day for Cheddar cheese (2). The FFA were eluted with 400 ml of Skellysolve B containing 1% butanol. This eluate was passed through a 4-g silicic acid column of McCarthy and Duthie (10). The column was washed, eluted as for butteroil, and the esters were prepared for gas-liquid chromatographic (GLC) analysis.

Preparation of butyl esters. A modification of the technique first suggested by Gander et al. (4) was employed. Five-tenths milliliter of n-butanol was added to the tube containing the dried salts, along with one drop of 0.03% methyl red in the same solvent. Concentrated sulfuric acid was added to the methyl red end point and 0.025 ml excess of sulfuric acid then added. The butyl esters of the fatty acids were formed by heating the sealed tube in a water bath at 100 C for 1.5 hr, after which anhydrous calcium sulfate was added and the mixture allowed to stand at room temperature for 45 min. The butyl alcohol solution was transferred as quantitatively as possible to a Babcock skim-milk test bottle. The tube was washed with 5 ml of 1% sodium bicarbonate solution, which was added to the Babcock bottle. The bottle was then shaken vigorously. Twenty per cent sodium chloride brine was added without agitation to bring the esters up to the neck of the bottle. The bottle was spun briefly in a Babcock centrifuge and the esters were brought into the neck of the bottle with brine.

Blank columns were run on each batch of freshly prepared solvents. The columns were developed exactly as described above, but in the absence of sample. The volume of the butanol in the neck of the Babcock bottle was adjusted to coincide with that of the sample.

Analysis of the butyl esters. The esters from the butteroil and cheese and the blanks were chromatographed on an Aerograph model 1520 dual-column instrument. Matched, packed columns were purchased from Wilkens Instrument and Research, Inc., Walnut Creek, California. The columns were 150 by .31 cm stainless steel and were packed with 15% diethyleneglycol succinate on 60/80- or 80/100-mesh chromosorb W treated with dimethyl dichlorosilane. The nitrogen carrier gas-flow rate was 24 ml/minute. The flame ionization detection system was used and the hydrogen flow rate was 30 ml/minute. Appropriate aliquots of the butyl alcohol solutions withdrawn directly from the necks of the Babcock bottles were injected into the GLC. It was necessary to run each butteroil sample twice; once at a high injection level to analyze the short-chain esters and once at a low injection level to analyze the long-chain esters. The temperature was programmed linearly from 40 to 190 C, the programming rate for the cheese esters was 4 C/minute until butyrate emerged, then the rate was increased to 8 C/minute. The programming rate for the butteroil esters was 4 C/minute. A cheese analysis was usually complete in 25 min, whereas a butteroil analysis took about 35 min. Esters were identified by their retention time compared to standard esters. The esters were quantified by triangulation and incompletely resolved peaks were treated as described by Peesok (12). Correction factors were derived as described by Bills et al. (3). The correction factors were based on the response of a given ester to that of the appropriate internal standard. Correction factors were obtained for 4:0, 6:0, and 8:0 compared to 7:0, and for 10:0, 12:0, 14:0, 16:0, 18:0, 18:1, 18:2, 18:3 compared to 17:0. A mixture of standard fatty acids consisting of 5–10 mg of each standard acid was dissolved in ether and applied to the silicic acid–KOH column and treated as before to obtain the butyl esters. Standard fatty acids from six separate columns were analyzed in duplicate to obtain correction factors.

A spread in weight ratios of acid to internal standard was obtained. When the above weight ratios were plotted against corresponding area ratios for each acid, a straight line which extra-

polated through the origin was obtained in each case. The slope of the line corresponded to the correction factor. It is apparent that the correction factors are not concentration-dependent.

Solubility test. The solubility of postassium salts of FFA was determined in the ether-methanol 1:1 v/v solvent. Forty milligrams of each acid, including 4:0, 6:0, 7:0, 8:0, 10:0, 12:0, 14:0, 16:0, 17:0, 18:0, 18:1, and 17 mg 18:3 were dissolved in 140 ml of ether and an equal volume of methanol added. The solution was titrated to the phenolphthalein end point with methanolic KOH. After standing overnight the solution remained clear, with no evidence of precipitation of the potassium salts. Therefore, this solvent system was used in the above analyses.

Completeness of extraction of FFA from cheese. The cheese cap material was isolated after the primary elution of FFA and was extracted three to six times each with 25 ml of Skellysolve B containing 1% butanol. This extract was treated as before, to isolate any residual FFA. Butyl esters of residual FFA were prepared and analyzed by GLC.

Saponification test. Six separate experiments were conducted to determine if any saponification of glycerides occurred during their passage through the basic column employed in this procedure. In three experiments 15 g of butteroil in 25 ml ether, and in three experiments 40 g of butteroil in 50 ml of ether were passed through 5 g silicic acid–KOH columns to remove endogenous FFA. This was repeated twice for the 15 g sample and three times for the 40 g sample. Each time the butteroil was washed from the column with 15 ml of ether. Each solution of butteroil in ether was again passed through a silicic acid–KOH column. The column was washed with 50 ml, then 100 ml of ether to remove the triglycerides, etc. The 15 g butteroil columns were then eluted with 50 ml of ether containing H_2SO_4 (1.4% v/v), followed by 100 ml ether. The 40 g butteroil columns were eluted with 50 ml of ether containing H_3PO_4 (2.5% v/v), followed by 100 ml ether. One hundred milliliters of ethanol were added to each sample and the solution titrated to the phenolphthalein end point with ethanolic KOH. After titration the samples were centrifuged and the supernatants concentrated as before. One milliliter of distilled water was added to the residue, the aqueous system acidified with concentrated HCl, and any FFA (or esters) removed by extracting three times each with 5 ml ether. The extracts were pooled in a tared 15 g centrifuge tube and evaporated to dryness under nitrogen. Traces of water were removed by storing in a desiccator over anhydrous calcium sulfate. The amount of residue obtained was compared to appropriate blanks run without butteroil. Presumably, the short-chain acids (or esters) would be lost during this procedure; however, the longer-chain acids (or esters) (10:0 and above) should be retained. If any esterification (see below) resulted in the sulfuric acid eluates, the long-chain esters as well as the long-chain acids (10:0 and above) should be carried through the procedure. The odor of butyric acid or ethyl butyrate was not detectable during these studies.

Esterification test. McGinnis and Dugan (11) found that, when a strong acid such as sulfuric acid was in contact with an alcohol and a fatty acid in an inert solvent at low temperature, esterification occurred. Tests were made in the present study to determine if any esterification occurred in the system containing the relatively weak phosphoric acid. In five replicate experiments, 25 mg of palmitic acid were dissolved in a small quantity of ether and placed on a 5 g silicic acid–KOH column. The sample was washed on to the column with 50 ml ether, then eluted with 50 ml phosphoric acid–ether as before, followed by 75 ml ether, titrated with ethanolic KOH after addition of 100 ml ethanol, centrifuged and concentrated as before. The residues were acidified with formic acid and taken into a minimal volume of carbon disulfide or ether and analyzed by GLC, using a 150 by .31 cm stainless steel column packed with 10% Carbowax 20 m-terephthalic acid on Fluoropak. The column temperature was programmed from 190 to 225 C[2], whereas the detector and injector temperatures were 275 and 222 C, respectively. Nitrogen and hydrogen flow rates were 30 ml/minute. The GLC pattern was compared to that observed for standard

[2] Although these temperatures are near the temperature maximum for Fluoropak, adequate data were obtained.

TABLE 1
Residue from silicic acid–KOH columns exposed to triglyceride compared to controls

Experiment	Sample	Control
	(mg)	
1[a]	3.9	4.2
2[a]	2.6	3.8
3[a]	1.7	1.5
4[b]	4.0	....
5[b]	7.9	4.0
6[b]	3.8	2.3

[a] Fifteen grams butteroil load.
[b] Forty grams butteroil load.

TABLE 2

Factors for relating the internal standards to the butyl esters of the other individual acids

Acid	No. of trials[a]	Average factor	Standard deviation
4:0	6	1.123	0.007
6:0	6	1.024	0.009
7:0	..	1.000[b]	.....
8:0	6	0.986	0.003
10:0	6	0.802	0.005
12:0	6	0.824	0.002
14:0	6	0.844	0.003
16:0	6	0.966	0.001
17:0	..	1.000[b]	.....
18:0	6	1.043	0.026
18:1	6	1.170	0.009
18:2	4	1.207	0.030
18:3	6	1.245	0.029

[a] Each trial run in duplicate.
[b] Assigned.

palmitic acid and ethyl palmitate. Under the above conditions, ethyl palmitate and palmitic acid are separated by a wide margin. Based on the GLC response to a known amount of ethyl palmitate, and the amount of sample injester onto the column, it was calculated that at least 1% esterification could be detected.

Results and Discusson

Esterification test. There was no detectable esterification of palmitic acid in the presence of phosphoric acid and ethanol based on the GLC experiments. Furthermore, the consistently high yields of butyrate and the very high degree of reproducibility of recovery for butyrate in the standards (Table 2) would indicate that esterification is not a problem.

Saponification test. Table 1 shows the residue resulting in tests to determine whether saponification occurs during the basic column treatment. These data indicate that there is as much residue from the control columns as from those which had been in contact with the triglyceride in the 15-g butteroil columns. With the 40 g butteroil columns, there was a very slight increase in the sample over control. The average amount of residue above the blanks was 1.8 mg. Assuming an average residue above the blank of 2 mg, and assuming that the saponification would reflect the composition of the milk fat and that saponification would be proportional to the amount of fat run through the column, some tentative calculations can be made. If butyric acid is 3.5% by weight in the milk fat, the amount of free butyric acid generated from 40 g of milk fat would be 0.07 or 1.7 mg per kilogram. This represents an error of approximately 18% when calculated from the lowest level of free butyric acid detected in the butteroil (9.3 mg/kg, Table3). On the other hand, this saponification error would be about 7% in the sample containing the highest level of butyric acid. For oleic acid and other long chain acids, the error would be even smaller. If the presence of FFA in butteroil were due to saponification on the column, the resultant FFA presumably should reflect the composition of milk fat. This is not the case, as evident from Table 3, where the short-chain fatty acids are relatively low and the long-chain fatty acids are relatively high in concentration.

The difference in residue weights could have been due to error; for example, residual FFA not completely removed by the pretreatment. However, assuming the worst, the error introduced by any saponification does not materially change results of these studies. It is rather obvious that any error introduced from examin-

TABLE 3

Averages of duplicate analyses of free fatty acids in three samples of butteroil[a] from three different sources

Acid	Butteroil I Weight FFA in fat	Butteroil I Deviation	Butteroil II Weight FFA in fat	Butteroil II Deviation	Butteroil III Weight FFA in fat	Butteroil III Deviation	Average deviation
	(mg/kg)	*(%)*	*(mg/kg)*	*(%)*	*(mg/kg)*	*(%)*	*(%)*
4:0	9.3	0.43	17.1	0.47	24.5	0.16	0.35
6:0	5.8	0.65	10.7	1.78	13.5	3.25	1.89
8:0	20.3	0.34	17.8	1.46	14.8	3.86	1.89
10:0	54.7	1.41	41.5	0.65	38.0	1.58	1.21
12:0	121.6	1.07	89.2	1.91	80.6	3.71	2.23
14:0	346.6	0.89	278.5	1.22	263.5	2.69	1.60
16:0	1,084.0	0.13	710.2	0.90	641.1	1.36	0.80
16:1	147.4	0.34	152.1	3.29	180.4	3.88	2.50
18:0	320.1	1.06	197.2	1.42	202.9	0.64	1.04
18:1	1,078.0	0.23	725.3	1.03	616.1	1.28	.84
18:2	107.3	0.84	102.8	2.53	77.9	0.35	1.24
18:3	38.0	1.26	40.2	0.72	20.9	1.34	1.11

[a] Butteroil was isolated by melting butter at 40 C, centrifuging and filtering the oil through sodium sulfate at 40 C.

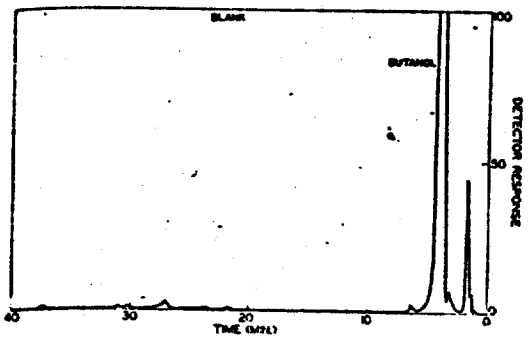

FIG. 1. Gas-liquid chromatogram of a blank sample. Programmed 40–190 C at 4 C/minute.

ing a 5 gram sample of cheese would indeed be negligible with the present technique.

Blank samples. The chromatograph of a blank sample shown in Figure 1 indicates that the interference from the solvents and column was negligible.

Correction factors. Table 2 lists the correction factors used to quantify the FFA content of the butteroil and cheese samples. Judging from the chromatograms and the correction factor, the recovery of butyrate was very good. The standard deviations of the correction factors are very small (Table 2). A chromatogram (Figure 2) of the standard butyl esters recovered from a silic acid–KOH column reveals quite good separation and an obviously high recovery of butyrate.

The above correction factors were applied in the same manner as by Bills et al. (3) to calculate the FFA content in three different samples of butteroil. The butteroil analyses are listed in Table 3. The butyric acid varied from 9.3 to 24.5 mg/kilogram butteroil. Although these values are slightly lower, the remaining

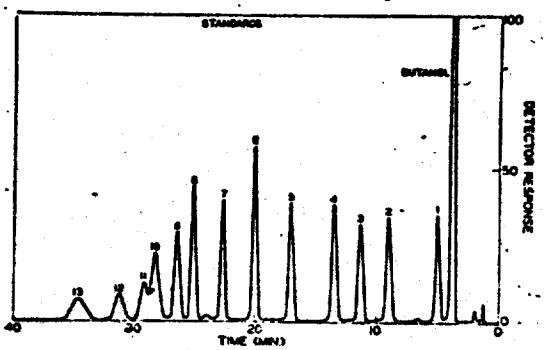

FIG. 2. Gas-liquid chromatogram for standard butyl esters recovered from silicic acid–KOH column.
1. 4:0 2. 6:0 3. 7:0 4. 8:0 5. 10:0 6. 12:0 7. 14:0 8. 16:0 9. 17:0 10. 18:0 11. 18:1 12. 18:2 13. 18:3.

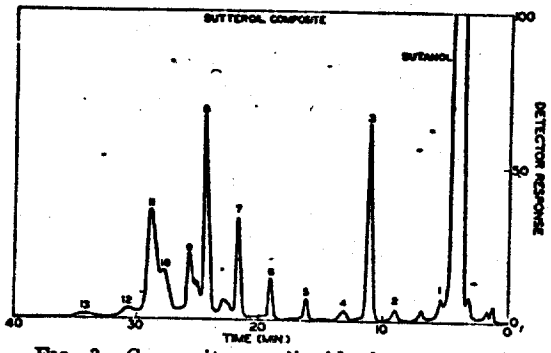

FIG. 3. Composite gas-liquid chromatogram of the separation of butyl esters from butteroil.
1. 4:0 2. 6:0 3. 7:0 4. 8:0 5. 10:0 6. 12:0 7. 14:0 8. 16:0 9. 17:0 10. 18:0 11. 18:1 12. 18:2 13. 18:3.

values seem to be of the same order of magnitude as reported by the above authors. Figure 3 shows a composite chromatogram of the separation of esters from butteroil. The esters from 4:0 through 8:0 were estimated at a high injection level, whereas the remaining esters were estimated at a low injection level. These chromatograms indicate that the short-chain esters are considerably lower in concentration than the long-chain esters. Perhaps this reflects the washing of butter in the butter-making process.

A chromatogram for the FFA in Provolone cheese (Figure 4) reveals a preponderance of the short-chain fatty acids. This is to be expected, because of the lipase treatment of the milk in the making process. The values in Table 4 reflect the very high concentrations of short-chain fatty acids. These values represent higher content of short-chain fatty acids than those reported for other cheeses. Analyses on

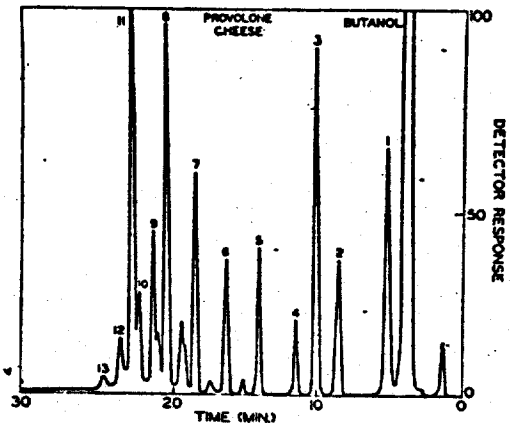

FIG. 4. Gas-liquid chromatogram of the FFA as butyl esters in Provolone cheese.
1. 4:0 2. 6:0 3. 7:0 4. 8:0 5. 10:0 6. 12:0 7. 14:0 8. 16:0 9. 17:0 10. 18:0 11. 18:1 12. 18:2 13. 18:3.

TABLE 4
Analysis of free fatty acids in Provolone cheese

Acid	Weight of acid in cheese I	II[a]
	(mg/kg)	
4:0	1,397.0	4,802.0
6:0	602.6	1,909.0
8:0	194.2	1,135.0
10:0	333.8	984.9
12:0	320.0	809.3
14:0	463.9	1,065.0
16:0	825.6	1,743.0
18:0	311.9	475.9
18:1	930.7	2,134.0
18:2	118.0	396.2
18:3	43.6	160.4

[a] Extra piccante type.

an extra piccante type of Provolone cheese indicate extremely high concentrations of short-chain fatty acids. Values for butyric acid are in the same general range as those reported by Harper and Long (5) and Long and Harper (9) for Provolone cheese.

Complete extraction of the FFA from cheese is apparently difficult. The chromatograms in Figure 5 indicate that small amounts of the longer-chain fatty acids may be recovered by a second, more exhaustive, extraction of the cheese cap from the silicic acid columns. It is not known to what extent these small amounts of FFA would affect the quantification of the FFA. Furthermore, if these FFA are not readily extractable, do they contribute to the flavor of the cheese?

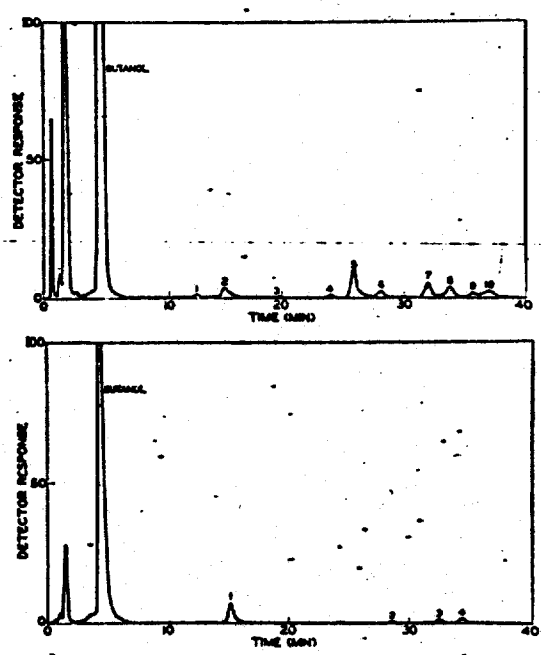

FIG. 5. Gas-liquid chromatograms of the butyl esters of the residual FFA in the cheese caps from the silicic acid columns.
Upper: 1. 7:0 2. 8:0 3. 10:0 4. 12:0 5. ? 6. 14:0 7. 16:0 8. 17:0 9. 18:0 10. 18:1.
Lower: 1. 8:0 2. 14:0 3. 16:0 4. 17:0.

The above technique is applicable to milk fat, cheese, and presumably any other product containing FFA. It should be no trouble to switch to methyl or other esters, if necessary.

Some advantages of the technique would be the direct determination of the FFA in the hexane-butanol extract, the high precision in determining the correction factors for short-chain fatty acids, the absence of a lengthy treatment and purification of the silicic acid, the absence of any necessity to work in a cold room, the absence of any specialized equipment or arbitrary technique for taking off low-boiling solvents. An analysis can be completed in about a day and a half; however, if one used BF_3 as a catalyst in the esterification, this could probably be cut to a day.

Acknowledgments

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057

Appendix B

to

CAPRYLIC ACID

A Monograph

Prepared for

Department of Health, Education and Welfare

Public Health Service

Food and Drug Administration

Rockville, Maryland

by

Dr. Paul C. Walter and

Dr. Steven J. Love

FIRL Project No. 80G-C3378-01

December 14, 1972

CAPRYLIC ACID

Hard copy of papers used in the summary of the monograph
(in order as they appear in the bibliography)

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Diabetes 16(11): 753-64 (1967)

Effect of Free Fatty Acids and Insulin on Protein Synthesis and Amino Acid Metabolism of Isolated Rat Diaphragms

24

Maria G. Buse, M.D., and John Buse, M.D., Charleston

SUMMARY

The effect of free fatty acids and insulin on the incorporation of C-14 from amino acids into muscle proteins was studied in vitro using "cut" or "intact" rat diaphragms.

Insulin promoted the incorporation of C-14 from L-glycine-2-C-14, L-histidine-2-ring-C-14 and L-leucine-U-C-14 into muscle proteins. Insulin stimulated the uptake of 0.05 mM. leucine-U-C-14 from the medium, but not its oxidation to C-14-O_2.

Palmitate (3 mM.) decreased the incorporation of C-14 from glycine and histidine into proteins of muscles stimulated with insulin. Octanoate (0.5-3 mM.) decreased the incorporation of C-14 from leucine into proteins. The inhibitory effect of octanoate on protein synthesis was demonstrated in the presence or absence of glucose and/or insulin. DL-β-hydroxybutyrate (15 mM.) decreased the incorporation of C-14 from leucine-U-C-14 into proteins in the presence of insulin.

Octanoate (1 mM.) stimulated the uptake of C-14 from leucine-U-C-14 from the medium and its oxidation to C-14-O_2. DL-β-hydroxybutyrate (15 mM.) appeared to inhibit both processes, however; the latter may be due to isotope dilution.

The nonutilizable amino acid, α-aminoisobutyric acid-C-14, penetrated less readily into the intracellular water of diaphragms incubated with 1 mM. octanoate than into controls. However, since octanoate stimulated the uptake of C-14 from leucine from the medium it appears likely that octanoate inhibited the incorporation of C-14 from leucine into proteins at a step beyond amino acid transport.

It is suggested that FFA can influence amino acid metabolism in muscles and decelerate their incorporation into proteins. Limitation of protein synthesis in muscle by increased intracellular FFA may contribute to the supply of amino acids available for neoglucogenesis during stress and carbohydrate deprivation. DIABETES *16*:753-64, November, 1967.

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From the Department of Medicine, Medical College of South Carolina, Charleston, South Carolina.

Glucose utilization in muscle is regulated in part by the intracellular concentration of free fatty acids (FFA). Increased FFA oxidation inhibits glycolysis. This phenomenon has been described as the glucose-fatty acid cycle and has been implicated as a cause of insulin resistance during prolonged fasting, following treatment with corticosteroids and growth hormone and in uncontrolled diabetes mellitus[1,2] With the exception of growth hormone treatment, the above conditions are accompanied by a negative nitrogen balance. The purpose of this study is to show that increased oxidation of FFA by muscle may influence amino acid metabolism in this tissue and may inhibit protein synthesis.

MATERIAL AND METHODS

Male rats of the Wistar strain, weighing 150 to 250 grams were used. In individual experiments the weight of the rats was as similar as possible, generally within 10 grams. The animals were fed ad libitum, (Wayne Lab blocks or Purina rat pellets) until they were sacrificed by a blow on the head. The diaphragms were removed immediately, rinsed free of blood with buffer, blotted and incubated. Three isolated diaphragm preparations were used. In the initial experiments on protein synthesis and in experiments concerning amino acid uptake and oxidation to CO_2 the two hemidiaphragms were dissected separately and incubated in separate vessels. This preparation will be referred to as the "cut" hemidiaphragm. In most of the experiments on protein synthesis and in studies of AIB transport the intact diaphragm preparation of Kipnis and Cori was used[3]; in most studies this preparation was divided along the midline.[4] The latter combines the advantage of minimizing the number of severed muscle fibers with the possibility of designing paired experiments. Thus, whenever "cut" or "intact hemidissected" diaphragms were used, one half was incubated with and the other without the fatty acid to be tested. Equal numbers of right and left half muscles were assigned to each medium.

The medium was Gey and Gey's balanced salt solution[5] (bicarbonate buffer) with the appropriate addi-

tions, equilibrated with O_2 and CO_2 (95:5), *pH* 7.4-7.5. Glucose when present in the medium was 1 mg. per milliliter and insulin* 1 or 100 mU. per milliliter. The incubation medium also contained a C-14 labeled amino acid (diluted as required with nonlabeled carrier), or other tracers such as sorbitol-H_3 and urea-C-14. Fatty acids (0.5-3 mM.) were added to alternate vessels. Octanoate and β-OH-butyrate were dissolved directly in the medium, following neutralization with equimolar NaOH. Palmitate was added to media containing crystalline bovine serum albumin (Armour) as a carrier. Palmitic acid was neutralized with a slight excess of NaOH, dissolved in a water bath at 60° C., and added dropwise to the medium with constant stirring.[6] In experiments involving palmitate, the control vessels (without added palmitate) also contained albumin. The FFA content of the albumin was measured by the method of Dole and Meinertz[7] and was 14.7 μmoles per gram.

The *pH* of the incubation media was measured before and at the end of the incubation period, by glass electrode, with the medium at 37.5° C., and at equilibrium with the gas phase. In some experiments the *pH* decreased slightly during incubation, generally less than 0.15 *pH*. There was no difference between the *pH* of control media and those containing FFA.

Following one-hour preincubation the tissues were gently blotted and transferred to vessels containing the incubation media. Generally, the preincubation media contained the same additions as the incubation media, except that the radioactive tracers and insulin were added to the latter only. Preincubation and incubation were in a Dubnoff metabolic shaker, at 37.5° C., with shaking (42 cycles per minute). Identical volumes were used for preincubation and incubation; these were 4 ml. medium for "cut" hemidiaphragms, 24 for with the intact diaphragm preparation and 12 ml. for its bisected modification. The purpose of preincubation was to alter the metabolic environment in the muscles (by increased fatty acid oxidation) before the amino acid tracer was added to the system.

Analytical procedures:

Tissue weight: When cut hemidiaphragms were used the tissues were weighed prior to incubation. The hemidiaphragms were placed into tared vessels containing the preincubation medium and reweighed to the nearest 0.1 mg. When the intact diaphragm or its bisected modification were used the hemidiaphragms were dissected free of the supporting tissues after incubation, rinsed for thirty seconds in buffer, blotted, placed into tared test tubes containing trichloroacetic acid (TCA), and weighed.

Incorporation of C-14 from amino acids into proteins: After two hours' incubation with a C-14 labeled amino acid the hemidiaphragms were placed into 5 ml. of 10 per cent TCA. Tissue proteins were isolated by TCA precipitation followed by extraction with hot TCA, performate digestion and reprecipitation with TCA.[8] In initial experiments the protein was suspended in a mixture of petroleum and ethyl ether (75:25) and placed on tared copper disks. The dried protein powder, aliquots of the medium and of the TCA supernatant were counted in a thin window gas flow counter. The planchets were reweighed and the counts corrected for self-absorption.

In later experiments, the dried protein powder was weighed and solubilized in Hyamine. One milliliter of Hyamine containing 3 to 5 mg. of protein, was added to 14 ml. of toluene scintillator* liquid and counted in a β-liquid scintillation counter. Aliquots of media before incubation (50λ) were also counted in the Hyamine-toluene-scintillator system. Counts were corrected for quenching with internal standards or by automatic external standardization. The radioactivity contained in the protein fraction was calculated as cpm per gram protein powder.

After incubation 0.2 ml. of each medium was added to 5 ml. of 10 per cent TCA. TCA extracts (0.5 ml.) of tissues and of media were added to 10 ml. of Bray's scintillation mixture* and counted. The nonprotein bound radioactivity in muscle was estimated by dividing the TCA soluble radioactivity contained per gram hemidiaphragm (wet weight) by the radioactivity contained per milliliter medium after incubation.

Studies concerning the transport of 2-aminoisobutyric acid-C-14 (AIB) and the extracellular space: Following one-hour incubation with AIB-C-14, sorbitol-H-3 and urea-C-14 hemidiaphragms were dissected free of supporting tissues, weighed and extracted with 2 ml. of boiling 5 per cent TCA for five minutes. TCA extracts of tissues and media after incubation were counted in Bray's scintillation mixture. The space of distribution of AIB, sorbitol or urea was expressed as the fraction of the wet weight of the tissue which would be at equilibrium with the medium at the end of incubation.

*Five times recrystallized beef insulin (25.4 U./mg.) was generously supplied by Dr. W. R. Kirtley, Lilly Research Laboratories, Indianapolis, Indiana.

*Five grams of 2,5-Diphenyloxazole (PPO) and 0.3 gm. of 1,4-bis-2 (4-Methyl-5-Phenyloxazolyl) benzene (dimethyl POPOP) dissolved in one liter of toluene.

$$Space = \frac{cpm/gm.\ tissue}{cpm/ml.\ medium} \times 100.$$

AIB-C-14 and sorbitol-H-3 spaces were measured simultaneously on the same tissue sample, by differential counting of the two isotopes in a three channel instrument. Quench corrections were made by external standardization.

Tissue water was determined as the space of distribution of urea-C-14 or as the difference between the wet weight and the dry weight of the tissues following incubation. Dry weight was obtained after drying in an oven at 110° C. for two hours. Tissue water was measured under identical conditions as the AIB spaces but in separate experiments.

Uptake of C-14 from leucine-C-14 and conversion to C-14-O_2: "Cut" hemidiaphragms were incubated in medicinal flasks sealed with rubber stoppers equipped with sleeves. Small plastic cups were attached with stainless steel wire to the inside of the stoppers. The flasks were gassed through a needle with O_2 and CO_2 (95:5) for five minutes at the beginning of incubation and sealed. After two hours 0.5 ml. of Hyamine was injected into the plastic cups and 0.5 ml. 10 N H_2SO_4 into the medium. The flasks were incubated for an additional hour at 37.5° C. The plastic cups containing the C-14-O_2 trapped in Hyamine were placed in counting vials containing 14 ml. of toluene scintillator mixture and counted. Aliquots of the incubation medium, obtained before and after incubation were also counted in the Hyamine-toluene system. C-14 uptake was calculated as the cpm that disappeared from the medium during incubation. Uptake and oxidation to CO_2 were related to the initial wet weight of the tissue.

Reagents: C-14 and H-3 labeled chemicals were purchased from the New England Nuclear Corporation and the Nuclear Chicago Corporation. Palmitic and octanoic acids (Grade A) were purchased from the Calbiochem Corporation; DL-β-hydroxybutyric acid (sodium salt) from the Sigma Corporation and Mann Research Laboratories. Fatty acids were stored at 4° C. protected from light and humidity. Octanoate was stored under a nitrogen atmosphere. Analytical reagents were used in all preparations.

Presentation of data: Duplicate samples were prepared and counted in all studies.

Means ± the standard errors of the means are tabulated. The *t* test of significance was carried out according to Fisher and Yates. The number of tissues used for each observation is indicated in parenthesis.

RESULTS

The effect of palmitate, albumin and insulin on protein synthesis (table 1)

Insulin stimulated the incorporation of C-14 from glycine and histidine into proteins of rat diaphragms ($p < 0.01$). This confirms studies of others. (For review of literature see references 10 & 11). Insulin increased the accumulation of nonprotein bound (TCA soluble) C-14 from glycine in the tissues ($p < 0.01$), but did not affect the accumulation of C-14 from histidine.[12]

Bovine serum albumin (2.5 gm. per cent) decreased the incorporation of C-14 from glycine into proteins and decreased the accumulation of TCA soluble C-14 from glycine in the tissues. These effects of albumin were significant ($p < 0.01$) only when protein synthesis was stimulated by the addition of insulin to the medium.

Albumin did not affect the incorporation of C-14 from histidine into proteins. However, the accumulation of TCA soluble C-14 from histidine in the tissues was significantly decreased by albumin in the presence or absence of insulin ($p < 0.01$). Albumin (1 gm. per cent) added to a medium containing insulin and leucine-U-C-14 did not affect the incorporation of C-14 into proteins, nor the accumulation of TCA soluble C-14 in the tissues. Inhibition of the incorporation of C-14 from glycine into proteins by 5 gm. per cent bovine serum albumin has been reported, and attributed to nonspecific amino acid competition.[13]

The addition of 3 mM. palmitate to a medium containing albumin decreased the incorporation of C-14 from glycine or histidine into proteins. This effect was not significant when the incubation was carried out without insulin in the medium. However, when 3 mM. palmitate was added to a medium containing insulin and albumin, the hemidiaphragms incorporated 10 to 20 per cent less C-14 glycine or histidine into proteins than controls incubated with albumin and insulin ($p < 0.05$).

Palmitate (1 mM.) added to a medium containing insulin and albumin had no significant effect on the incorporation of C-14 from leucine-U-C-14 into proteins. The concentration of nonprotein bound C-14 from leucine-U-C-14 was slightly but significantly greater ($p < 0.01$) in diaphragms incubated with 1 mM. palmitate, albumin and insulin than in controls incubated with albumin and insulin.

The effect of octanoate and insulin on protein synthesis (table 2)

TABLE 1

The effect of palmitate on the incorporation of C-14 from amino acids into proteins of isolated rat diaphragms

Experiment	Diaphragm preparation	Amino acid μM.		Additions to medium* Albumin mg/ml.	FFA mM.	Insulin mU./ml.	C-14 incorporation into proteins mμg./gm. protein†	FFA effect p‡	Nonprotein bound C-14 in muscle "Space" ± S.E.M.§
A	"Cut"	L-glycine-2-C-14 2 μM.	a	—	—	—	5.94 ± 0.46 (6)		240 ± 11.1 (6)
			b	25	—	—	5.22 ± 0.32 (6)	c < b	218 ± 7.1 (6)
			c	25	3	—	4.88 ± 0.16 (6)	n.s.	207 ± 7.0 (6)
			d	—	—	100	10.34 ± 0.16 (6)		370 ± 22.0 (6)
			e	25	—	100	9.18 ± 0.24 (6)	f < e	282 ± 11.1 (6)
			f	25	3	100	8.26 ± 0.28 (6)	< 0.05	287 ± 15.9 (6)
B	"Cut"	L-histidine 2-ring-C-14 140 μM.	a	—	—	—	424.0 ± 30.8 (8)		421 ± 38.7 (8)
			b	25	—	—	416.0 ± 14.0 (8)	c < b	334 ± 11.8 (8)
			c	25	3	—	389.0 ± 30.8 (8)	n.s.	290 ± 19.6 (8)
			d	—	—	100	529.0 ± 29.4 (8)		450 ± 21.5 (8)
			e	25	—	100	519.0 ± 21.0 (8)	f < e	340 ± 23.8 (8)
			f	25	3	100	434.0 ± 18.0 (8)	< 0.05	323 ± 30.9 (8)
C	"Intact bisected"	L-leucine-U-C-14 50 μM.	d	—	—	1	583.0 ± 48.5 (8)		91 ± 3.5 (12)
			e	12.5	—	1	616.0 ± 33.5 (11)	f < e	86 ± 2.3 (18)
			f	12.5	1	1	589.0 ± 26.5 (11)	n.s.	97 ± 4.4 (18)

*Diaphragms were preincubated (1 hr.) and incubated (2 hrs.) in balanced salt solutions containing glucose (1 mg./ml.). Albumin and palmitate were added during both phases; insulin and amino acids only during the incubation period. The amino acid concentrations indicated include the labeled amino acid (0.05-0.2 μc./ml.) and the nonlabeled carrier.
†cpm/gm. tissue protein × amino acid mμmoles/cpm in medium before incubation.
‡p values for insulin effect on C-14 incorporation into proteins. In study A: d > a, e > b, f > c = each p < 0.01; (e-b) > (f-c) = n.s. In study B: d > a, e > b = each p < 0.01. f > c = n.s. (e-b) > (f-c) = n.s. Albumin effect: only significant difference c < d (p < 0.01) in study A.
§TCA sol. cpm/gm. tissue / cpm/ml. medium after incubation.
Significant p values in "spaces": In study A: d > a, e > b, f > c (each p < 0.01, insulin effect). e < d (p < 0.01, albumin effect). In B: b < a, e < d, (each p < 0.05, albumin effect). In C: f > e (p < 0.01, FFA effect).

As reported previously[10,11,14] the incorporation of C-14 from leucine into proteins was significantly stimulated by the addition of 1 mU. of insulin to the medium ($p < 0.01$).

The addition of octanoate to an insulin containing medium significantly decreased the incorporation of C-14 from leucine into proteins ($p < 0.01$ in studies A-G). This was demonstrated with "cut" hemidiaphragms and 3 mM. octanoate (study A) and with the "intact diaphragm" preparation and 0.5 or 1 mM. octanoate (studies B-G). It occurred in the presence of glucose in the medium (studies A, B, D-G) and in its absence (study C). Although insulin significantly stimulated protein synthesis in the presence of octanoate in study A ($p < 0.05$), paired analysis of the data showed that the effectiveness of insulin was diminished by octanoate ($p < 0.05$).

In the absence of insulin, octanoate decreased the C-14 incorporated into proteins slightly. This effect was not significant when the medium contained glucose in addition to FFA (studies A & B). However, in study C, which was carried out without glucose in the medium, 1 mM. octanoate significantly inhibited the incorporation of C-14 from leucine into proteins even in the absence of insulin ($p < 0.01$).

The concentration of leucine in the medium was 0.05 mM. in each study in table 2, except F and G. (The leucine content of rat serum has been reported as 0.16 mM.)[11]

Leucine was increased to 0.5 mM. in study F to ascertain whether or not the effect of octanoate on C-14 incorporation into proteins could represent isotopic dilution, instead of inhibition of protein synthesis. During incubation with 1 mM. octanoate-1-C-14 approximately 0.7 per cent of the C-14 which was taken up by the diaphragms was incorporated into protein (table 3). This represented 0.1 μmole of octanoate per gram tissue in two hours or 0.5 μmole per gram protein. Fragments of octanoate molecules may have entered the leucine pool by transamination. During incubation with 0.05 mM. leucine-U-C-14, approximately 50 per cent of the radioactivity that disappeared from the medium was incorporated into proteins (compare table 5 and table 2). This represented 0.5 to 1.2 μmoles of leucine per gram

TABLE 2

The effect of octanoate on the incorporation of C-14 from leucine into proteins

Experiment	Diaphragm preparation	Control medium* Leucine μM. (label)	Glucose mg./ml.	Additions	C-14 incorporations into proteins: mμmoles/gm. protein† Mean ± S.E.M.	FFA effect d ± S.E.M.	p‡
A	"Cut"	50 (U-C-14)	1	a Control	800± 89 (7)		
				b + 3 mM. FFA	705± 39 (7)	— 95	n.s.
				c + insulin	1,205±131 (6)		
				d + FFA + insulin	850± 51 (7)	—355± 92	<0.01
B	"Intact"	50 (U-C-14)	1	a Control	530± 41 (8)		
				b + 1 mM. FFA	460± 67 (8)	— 70	n.s.
				c + insulin	870±107 (8)		
				d + FFA + insulin	635±117 (8)	—235± 61	<0.01
C	"Intact bisected"	50 (U-C-14)	—	a Control	585± 20 (12)		
				b + 1 mM. FFA	495± 30 (12)	— 90± 22	<0.01
				c + insulin	765± 79 (12)		
				d + FFA + insulin	595±163 (12)	—170± 40	<0.01
D	"Intact bisected"	50 (U-C-14)	1	c Control + insulin	1,045± 70 (14)		
				d + 0.5 mM. FFA + ins.	865± 66 (14)	—180± 34	<0.01
E	"Intact bisected"	50 (1-C-14)	1	c Control + insulin	525± 41 (9)		
				d + 1 mM. FFA + ins.	400± 28 (10)	—125± 58	<0.01
F	"Intact bisected"	500 (U-C-14)	1	c Control + insulin	3,250±130 (10)		
				d + 1 mM. FFA + ins.	2,350± 75 (10)	—900±130	<0.01
G	"Intact bisected"	500 (U-C-14) No preincubation	1	c Control + insulin	1,950±120 (10)		
				d + 1 mM. FFA + ins.	1,550±110 (10)	—400± 95	<0.01

*Diaphragms were preincubated (1 hr.) and incubated (2 hrs.) in balanced salt solution with the indicated additions. Octanoate and glucose were added during both phases, leucine-C-14, carrier leucine and insulin (1 mU./ml. when present) during incubation only. Leucine-C-14 = 0.05 μc./ml. = 0.25 μM. (Spec. act. > 200 mc./mmole). The concentrations of carrier leucine are indicated.

†cpm/gm. tissue protein × leucine mμmoles/cpm in medium before incubation.

‡p = paired analysis. p values for insulin effects: in study A: c > a = p < 0.01: d > b = p < 0.05; (c-a) > (d-b) = p < 0.05. In study B: c > a = p < 0.01, d > b = n.s.; (c-a) > (d-b) = n.s. In study C the effect of octanoate in the presence or absence of insulin was studied in separate experiments.

protein in two hours. The inhibition of protein synthesis observed in the presence of 1 mM. octanoate was in the order of 15 to 30 per cent. Therefore, an isotope dilution effect could not be ruled out completely.

Study F in table 2 disproves this hypothesis. The concentration of leucine was 0.5 mM. and 3.2 μmoles of leucine were incorporated per gram protein in two hours. The addition of 1 mM. octanoate to the medium decreased the incorporation of C-14 from leucine into proteins by 0.9 μmoles per gram protein. A tenfold increase in the leucine concentration of the medium did not diminish the inhibitory effect of FFA on the incorporation of C-14 from leucine into proteins suggesting that the octanoate induced inhibition is exerted on protein synthesis per se.

In every study except G in table 2 the two-hour incubation period in the presence of the radioactive tracer was preceded by one-hour preincubation with all the ingredients to be tested except the C-14 labeled amino acid, its nonlabeled carrier and insulin. Study G was carried out under identical conditions as study F except that the period of preincubation was omitted. The diaphragms which had not been preincubated incorporated less C-14 from leucine into proteins (study G) than the diaphragms which had been preincubated for one hour without the radioactive tracer (study F). The two studies were carried out several months apart and this difference may be due to a noncontrolled variable. However, it is possible that the endogenous stores of leucine decreased during the one-hour preincubation resulting in heavier labeling of the newly formed protein during the subsequent incubation period. Although this point is not resolved, study G shows clearly that preincubation is not a prerequisite for the inhibitory effect of octanoate on protein synthesis. Diaphragms which were not preincubated incorporated 23 per cent less C-14 into proteins in the presence of octanoate than the controls ($p < 0.01$), while octanoate decreased C-14 incorporation by 28 per cent in the preincubated series ($p < 0.01$).

TABLE 3

Metabolism of C-14 from octanoate-1-C-14 by diaphragms

		μmoles/gm. tissue/2 hrs.
(A)	C-14 uptake from medium	14.01±0.44 (8)
(B)	Oxidation to C-14-O_2	4.29±0.17 (8)
(C)	Incorporation into protein	0.10±0.004 (4)

The medium was balanced salt solution[5] containing 1 mM. nonlabeled octanoate during preincubation (1 hr.); octanoate-1-C-14 (0.25 μc./ml.) with nonlabeled octanoate (final conc. 1 mM.) and 5×10^{-2} mM. nonlabeled leucine during incubation (2 hrs.). A and B were determined simultaneously, using "cut" hemidiaphragms and 4 ml. of medium. "Intact hemidissected" diaphragms were incubated in 12 ml. of medium in C. Results in C were originally determined per milligram protein. The calculation assumes that protein constitutes 18 per cent of the weight of the tissue.

The effect of DL-β-hydroxybutyrate on protein synthesis (table 4)

DL-β-hydroxybutyrate was less effective than octanoate in inhibiting protein synthesis. In preliminary experiments (not shown in table 4), 1 or 4 mM. DL-β-hydroxybutyrate did not affect the incorporation of C-14 from leucine into proteins. DL-β-hydroxybutyrate decreased the incorporation of C-14 from leucine into proteins when added at a 15 mM. concentration to media containing insulin. The inhibition was of borderline significance ($0.05 < p < 0.1$) when glucose was also present in the medium, but was significant in the absence of glucose ($p < 0.01$). In the absence of insulin, β-hydroxybutyrate did not affect the incorporation of C-14 into proteins.

The effect of octanoate, β-hydroxybutyrate and insulin on leucine uptake and oxidation

C-14 uptake and oxidation were studied during incubation with 0.05 mM. leucine-U-C-14 (table 5). Insulin stimulated the disappearance of C-14 from leucine-U-C-14 from the medium ($p < 0.01$).[14] Insulin did not affect the oxidation of leucine-U-C-14 since the cpm recovered in C-14-O_2 were essentially identical during incubation with and without insulin. Presumably the extra leucine-C-14 that was taken up by the diaphragm under the influence of insulin was incorporated into proteins and not made available for oxidation.

Ocanoate 1 mM., added to the medium in the absence of insulin, caused the diaphragms to utilize 40 per cent more C-14 from the medium than the controls ($p < 0.01$) and increased the oxidation of C-14 from leucine by 200 per cent ($p < 0.01$). While in control muscles 15 to 20 per cent of the C-14 that disappeared from the medium was recovered as C-14-O_2, in the presence of octanoate the oxidized fraction was doubled. Although octanoate appeared to enhance the uptake of C-14 from leucine more than insulin, this difference was not significant. Octanoate added to an insulin containing medium caused no significant further stimulation of leucine uptake. However, octanoate in the presence of insulin increased the fraction of C-14 from leucine that was oxidized by 160 per cent ($p < 0.01$).

The effect of β-hydroxybutyrate was the opposite of that of octanoate. β-hydroxybutyrate decreased the uptake of C-14 from leucine-U-C-14 by 18 to 19 per cent in the absence and in the presence of insulin ($p < 0.01$). It

TABLE 4

The effect of DL-β-hydroxybutyrate on the incorporation of C-14 from leucine-U-C-14 into proteins

Experiment		Additions to medium* Glucose 1 mg./ml.	DL-β-OH-butyrate 15 mM.	Insulin 1 mU./ml.	C-14 incorporation into proteins: mμmoles/gm. protein† Mean ± S.E.M.	FFA effect d ± S.E.M.	p‡
A	a	+	—	—	615± 30 (8)		
	b	+	+	—	670± 51 (8)	+ 55	n.s.
	c	+	—	+	945± 80 (7)		
	d	+	+	+	840±106 (7)	—105±49	<0.1
B	a	—	—	—	505± 55 (14)		
	b	—	+	—	555± 74 (14)	+ 50	n.s.
	c	—	—	+	680± 33 (12)		
	d	—	+	+	555± 47 (12)	—125±36	<0.01

"*Intact bisected" diaphragm preparations were preincubated (1 hr.) and incubated (2 hrs.) in balanced salt solution[5] with the indicated additions. Insulin, leucine and leucine-U-C-14 were added only during the incubation period. Leucine-U-C-14 = 0.05 μc./ml. = 0.25 μM. Nonlabeled carrier leucine = 50 μM.

† cpm/gm. tissue protein × leucine mμmoles/cpm in medium before incubation.

‡ p values for insulin effect. In Study A: c > a = p < 0.01, d > b = n.s. In Study B: c > a = p < 0.02, d = b (n.s.)

also caused a 50 per cent decrease in the oxidation of C-14 from leucine-U-C-14 ($p < 0.01$).

The effect of octanoate, β-hydroxybutyrate and insulin on the accumulation of TCA-soluble C-14 from leucine in rat diaphragms (table 6)

In each experiment where the tracer used was 0.05 mM. uniformly labeled leucine-C-14 the addition of octanoate to the medium promoted the accumulation of TCA soluble radioactivity in the tissues ($p < 0.01$, studies A-D). This occurred in the presence or in the absence of insulin; with "cut" or "intact" diaphragms and with concentrations of octanoate varying from 0.5 to 3 mM. When the concentration of leucine in the medium was increased to 0.5 mM., the addition of octanoate did not affect the accumulation of TCA soluble C-14 from leucine-U-C-14 in the tissues (study E).

While octanoate enhanced the accumulation of TCA soluble C-14 from leucine-U-C-14 in the tissues, β-hydroxybutyrate decreased it. In each study where the tracer was 0.05 mM. leucine-U-C-14, less TCA soluble radioactivity accumulated in the tissues incubated with β-hydroxybutyrate than in the controls. This occurred in the presence or absence of insulin and/or glucose in the medium (studies F and G, $p < 0.01$).

Manchester has shown that although rat diaphragms oxidize a considerable portion of the leucine skeleton, C-1 is oxidized preferentially.[15] It appeared probable that the difference in the TCA soluble C-14 content of hemidiaphragms incubated with octanoate or β-hydroxybutyrate reflected a difference in the concentration of C-14 labeled fragments of the leucine-U-C-14 molecule which accumulated during leucine oxidation.

To test this hypothesis hemidiaphragms were incubated with octanoate or β-hydroxybutyrate and 0.5 mM. leucine-1-C-14 instead of leucine-U-C-14 as a tracer (study H). Under these conditions the effects of octanoate and β-hydroxybutyrate on the accumulation of TCA soluble C-14 were reversed. Octanoate significantly decreased the accumulation of TCA soluble C-14 from leucine-1-C-14 ($p < 0.01$) while β-hydroxybutyrate increased it ($p < 0.01$). This suggests that the concentration of TCA soluble C-14 from leucine-1-C-14 in the muscles was inversely proportional to the rate of oxidation of leucine.

During incubation with leucine-1-C-14 and insulin but without FFA the space of distribution of nonprotein bound C-14 in the tissues was 33 per cent less than the "spaces" obtained with leucine-U-C-14 under identical experimental conditions. The difference presumably represents the accumulation of C-14 labeled fragments of leucine-U-C-14 in the tissue water. Akedo and Christensen incubated diaphragms with 0.5 mM. leucine-C-14 and reported that more than 90 per cent of the nonprotein bound radioactivity in the tissues belonged to the unaltered amino acid.[12] They did not specify the position of the label on the leucine molecule. The apparent space

TABLE 5

The effect of fatty acids on the uptake and oxidation of C-14 from leucine-U-C-14 by isolated rat diaphragms

		FFA	mM.	Insulin mU./ml.	C-14 uptake* mμmoles/gm. tissue	FFA effect p‡	C-14-O_2 production mμmoles/gm. tissue†	FFA effect p‡	C-14-O_2 × 100 / C-14 uptake
A	a	Octanoate	—	—	210± 7 (8)		41±0.2 (8)		19.5
	b		1	—	291±11 (8)	↑p<0.01	123±4.7 (8)	↑p<0.01	42.3
	c		—	1	263±16 (8)		35±2.1 (8)		13.3
	d		1	1	296±17 (8)	↑n.s.	103±7.9 (8)	↑p<0.01	34.8
B	a	DL-β-OH-	—	—	228± 6 (4)		34±4.0 (4)		15.0
	b	butyrate	15	—	188± 5 (4)	↓p<0.01	15±0.2 (4)	↓p<0.01	8.1
	c		—	1	340±20 (8)		33±2.1 (8)		9.7
	d		15	1	274±20 (8)	↓p<0.05	16±0.1 (8)	↓p<0.01	5.9

Hemidiaphragms were incubated for two hours in 4 ml. of balanced salt solution containing glucose 1 mg./ml., L-leucine-U-C-14 (50 mμmoles/ml. = 0.1 μc./ml.), and the additions indicated in the table. Preincubation (1 hr.) was in identical media, but without leucine-C-14 or insulin.

*C-14 uptake = 4 (cpm/ml. IM—cpm/ml. FM) /gm. tissue/2 hrs. × leucine mμmoles/cpm IM, where IM is the initial medium and FM the medium after 2 hrs. incubation.

†C-14-O_2 production = cpm/gm. tissue/2 hrs. × leucine mμmoles/cpm in medium before incubation.

‡p values for insulin effect. In C-14 uptake, Study A: c > a = p < 0.01, d > b = n.s. In Study B: c > a = p < 0.01, d > b = p < 0.01. In C-14-O_2 production = no significant insulin effect.

TABLE 6

The effect of octanoate and DL-β-hydroxybutyrate on the accumulation of nonprotein bound C-14 from leucine in isolated rat diaphragms

Experiment	Control medium *Leucine μM. (label)	Glucose mg./ml.	Additions	Nonprotein bound C-14 in muscle "Space" ± S.E.M.†	FFA effect p‡
A	50 (U-C-14)	1	a Control	119± 6.2 (7)	
			b + 3 mM. octanoate	151± 4.9 (7)	↑p<0.01
			c + insulin	94± 6.4 (7)	
			d + octanoate + insulin	150± 8.4 (7)	↑p<0.01
B	50 (U-C-14)	1	a Control	114± 5.7 (8)	
			b + 1 mM. octanoate	141±11.2 (8)	↑p<0.05
			c + insulin	84± 2.3 (8)	
			d + octanoate + insulin	120±10.0 (8)	↑p<0.01
C	50 (U-C-14)	—	a Control	100± 3.2 (12)	
			b + 1 mM. octanoate	140± 3.8 (12)	↑p<0.01
			c + insulin	91± 2.8 (10)	
			d + octanoate + insulin	137± 3.3 (10)	↑p<0.01
D	50 (U-C-14)	1	c Control + insulin	87± 1.9 (15)	
			d + 0.5 mM. octanoate + insulin	128± 3.7 (15)	↑p<0.01
E	500 (U-C-14)	1	c Control + insulin	131± 3.5 (10)	
			d + 1 mM. octanoate + insulin	127± 5.7 (10)	n.s.
F	50 (U-C-14)	1	a Control	117± 5.7 (8)	
			b + 15 mM. DL-β-OH-butyrate	80± 1.6 (8)	↓p<0.01
			c + insulin	89± 3.3 (8)	
			d + β-OH-butyrate + insulin	75± 1.8 (8)	↓p<0.01
G	50 (U-C-14)	—	a Control	122± 3.6 (14)	
			b + 15 mM. DL-β-OH-butyrate	99± 3.1 (14)	↓p<0.01
			c + insulin	95± 2.0 (12)	
			d + β-OH-butyrate + insulin	79± 1.6 (12)	↓p<0.01
H	50 (1-C-14)	1	c Control + insulin	60± 1.4 (8)	
			d + 1 mM. octanoate + insulin	51± 1.4 (8)	↓p<0.01
			e + 15 mM. DL-β-OH-butyrate + insulin	73± 1.2 (8)	↑p<0.01

*L-leucine-U-C-14 or 1-C-14 = 0.05 μc/0.25 μM. The concentrations of carrier leucine are shown. The tissues were preincubated for one hour and incubated for two hours in balanced salt solution with the indicated additions. Leucine-C-14, carrier leucine and insulin (1 mU./ml. when present) were added only during incubation. "Cut" hemidiaphragms were used in study A, "intact diaphragms" in B, and "intact bisected" preparations in C-H.

$$\dagger \text{Space} = \frac{\text{TCA sol. cpm/gm. tissue}}{\text{cpm/ml. medium after incubation}} \times 100.$$

‡p values for insulin effects. In studies A, B, C, F, and G: c < a = (in each $p < 0.01$). d < b = n.s. in studies A, B, C and F; $p < 0.01$ in G.

of distribution reported by these authors is similar to that observed in study H using 0.05 mM. leucine-1-C-14, and much lower than that observed using 0.5 mM. leucine-U-C-14 in study E.

Insulin without added FFA significantly decreased the accumulation of TCA soluble C-14 in diaphragms incubated with 0.05 mM. leucine-U-C-14 ($p < 0.01$ in studies A, B, C, F & G). In the presence of octanoate, insulin did not affect the accumulation of C-14 from leucine-U-C-14 in the diaphragm (Studies A-C).

The effect of octanoate and β-hydroxybutyrate on AIB transport

Since diaphragms incubated with octanoate took up more leucine-C-14 from the medium than the controls, the inhibitory effect of octanoate on the incorporation of C-14 from leucine into proteins cannot be ascribed to

decreased penetration of the amino acids into muscle cells. Indeed, it is necessary to postulate that octanoate influenced amino acid metabolism primarily beyond the transport step into muscle cells. The effect of octanoate and β-hydroxybutyrate on the penetration of a nonutilizable amino acid, α-aminoisobutyric acid-3-C-14 (AIB) into muscle cells is shown in table 7. The transport of AIB is known to be accelerated by insulin.[12,16]

Diaphragms incubated with 3 mM. octanoate showed a small but significant increase in the space of distribution of sorbitol and a slight increase in total tissue water. This suggests that high concentration of octanoate interfered with the integrity of the cell membrane. In addition 3 mM. octanoate inhibited the stimulatory effect of insulin on the transport of AIB ($p < 0.01$).

Incubation with 1 mM. octanoate did not affect the space of distribution of sorbitol or the water content of the diaphragms. Diaphragms incubated with 1 mM. octanoate responded to insulin with stimulation of AIB penetration, which is further proof of the relative integrity of the cell membrane. Octanoate (1 mM.) decreased the penetration of AIB into the tissue water of muscle cells by approximately 20 per cent in diaphragms incubated with or without insulin ($p < 0.01$, paired analysis).

DL-β-hydroxybutyrate (15 mM.) had no effect on the space of distribution of sorbitol or on the water content of the diaphragms. However, β-hydroxybutyrate increased the concentration of C-14 from AIB in muscle cells significantly, in the absence of insulin and when the transport of AIB was stimulated by insulin ($p < 0.01$, paired analysis).

C-14-O_2 production from AIB-C-14 (or from contaminants of the preparation) was measured in one experiment. "Cut" hemidiaphragms incubated without insulin or FFA oxidized 4.3 per cent of the C-14 contained per milliliter medium per gram tissue per hour when the AIB concentration was 8.5×10^{-3} mM. (Under the same conditions hemidiaphragm oxidized 82 per cent of the radioactivity contained per milliliter medium per gram tissue per two hours when the tracer was 5×10^{-2} mM. leucine-U-C-14). The addition of octanoate (1 mM.) or β-hydroxybutyrate (15 mM.) decreased the oxidation of AIB-3-C-14 (or its labeled contaminant) from 0.36 mμmoles per gram tissue per hour to 0.21 mμmoles per gram tissue per hour. The effects of the two FFA on oxidation were essentially the same. Since 1 mM. octanoate decreased the concentration of C-14 from AIB in the intracellular water, and reduced the small oxidized fraction of AIB-C-14, it is probable

TABLE 7

The effect of octanoate and β-hydroxybutyrate on the penetration of AIB into rat diaphragms

	FFA	μmoles/ml.	Glucose mg./ml.	Insulin mU./ml.	AIB space* Per cent of wet wt. ± S.E.M.	Sorbitol space* Per cent of wet wt. ± S.E.M.	Tissue water* Per cent of wet wt. ± S.E.M.	"Intracellular AIB space"†
A	Octanoate	—	1	—	73.3±3.5 (8)	27.3±0.7 (8)	78.5±0.2 (8)	89.7
		3	1	—	81.3±3.3 (8)	32.6±1.2 (8)	80.7±1.0 (8)	101.3
		—	1	1	124.2±3.9 (8)	28.0±1.3 (8)	77.6±0.4 (8)	193.9
		3	1	1	93.0±7.4 (8)	33.6±2.3 (8)	79.2±0.4 (8)	130.2
B	Octanoate	—	1	—	57.7±2.6 (12)	24.4±3.1 (12)	75.2±1.1 (10)	65.6
		1	1	—	51.0±2.0 (12)	24.7±1.1 (12)	76.2±1.0 (10)	50.9
		—	1	1	85.0±5.7 (12)	23.3±0.6 (12)		119.0
		1	1	1	73.4±4.2 (12)	25.8±1.5 (12)		94.5
C	DL-β-hydroxybutyrate	—	—	—	53.6±2.4 (8)	24.2±1.2 (8)	75.2±1.1 (10)	57.8
		15	—	—	62.7±3.5 (8)	23.3±1.6 (8)	74.0±1.2 (10)	77.7
		—	—	1	90.1±5.2 (8)	26.1±1.8 (8)		140.3
		15	—	1	111.3±8.1 (8)	26.0±2.5 (8)		177.8

Intact diaphragms were used in study A, and "Intact hemidissected" in B and C. AIB = α-aminoisobutyric acid-3 C-14, 8.5×10^{-3} mM. (0.04 μc./ml.). Sorbitol-H^3 = 6×10^{-3} mM. (0.5 μc./ml.). The Sorbitol and AIB spaces were measured simultaneously in studies B and C, but not in A. Tissue water was determined in separate experiments; as wet weight—dry weight in A, and as the space of distribution of urea-C-14 in B and C. Preincubation (1 hr.) and incubation (1 hr.) were in balanced salt solution[5] with the additions as indicated. FFA and glucose were added during both phases, insulin and labeled tracers only during incubation.

*AIB, Sorbitol and urea spaces were calculated as $= \frac{\text{cpm/gm. tissue}}{\text{cpm/ml. final medium}} \times 100.$

†Calculated as $= \frac{\text{AIB space—Sorbitol space}}{\text{100—Sorbitol space}}$ where space $= \frac{\text{cpm/ml. tissue water}}{\text{cpm/ml. final medium}} \times 100.$

that octanoate exerted an inhibitory effect on AIB transport. The increased concentration of C-14 from AIB in the intracellular water of diaphragms incubated with 15 mM. β-hydroxybutyrate may reflect accelerated transport of AIB, or may be a result of decreased C-14 oxidation.

DISCUSSION

There is increasing evidence that fatty acids (or products of their metabolism) can affect various metabolic processes in vitro. They inhibit glucose utilization by muscle and fatty acid synthesis by the liver. The inhibitory effect of FFA on glucose utilization is exerted at several key metabolic steps: (a) inhibition of the transport of glucose across the muscle cell membrane, (b) deceleration of glycolysis via inhibition of phosphofructokinase and secondarily of hexokinase, (c) inhibition of pyruvate oxidation (for review of literature see references 1 & 2). In liver, long-chain acyl CoA derivatives competitively inhibit glucose-6-P-dehydrogenase.[17] Free fatty acids and long-chain acyl CoA derivatives inhibit hepatic acetyl CoA carboxylase. A negative feedback mechanism operating at this step has been suggested as a major site of regulation of fatty acid synthesis (for review of literature see references 18 & 19). Kalkhoff and Kipnis reported that the in vitro addition of 1 to 5 mM. palmitate to cell-free homogenates of rat liver did not affect protein synthesis, although FFA synthesis was inhibited.[18]

The data presented indicate that FFA, or byproducts of FFA metabolism can inhibit muscle protein synthesis in vitro. The inhibitory effect of FFA on the incorporation of C-14 from amino acids into muscle proteins was demonstrated with three different amino acids and three fatty acids, using "cut" or "intact" diaphragm preparations. The inhibitory effect of each FFA studied was evident when protein synthesis was stimulated by insulin; octanoate (1 mM.) caused significant inhibition also in the absence of insulin when glucose was omitted from the medium. The absence of glucose apparently potentiated the inhibitory effect of octanoate on protein synthesis, although octanoate was used as an energy source by the diaphragm (table 3). This may be due to the decreased availability of α-glycerophosphate for the esterification of FFA in muscle cells deprived of glucose.

Inhibition of protein synthesis by FFA was not secondary to inhibition of glycolysis, since it was demonstrated with or without glucose in the medium. The one-hour preincubation in a glucose-free medium assured that in experiments without glucose, this substrate was virtually absent from the extracellular space at the time the C-14 amino acid was added to the system and the muscle's glycogen stores were virtually depleted.[3]

Our data support the findings of Johnson et al.[20] and Manchester[13] indicating that diaphragms oxidize leucine and that C_1 is oxidized preferentially.[13] The present study suggests that leucine uptake and oxidation by striated muscle is influenced by fatty acids. Octanoate stimulated leucine uptake and oxidation while DL-β-hydroxybutyrate appeared to inhibit both parameters. β-hydroxybutyrate is an intermediate metabolite of leucine.[20] Therefore, the decreased C-14-O_2 production from leucine-U-C-14 during incubation with β-hydroxybutyrate may have been due to excess β-hydroxybutyrate competing with β-hydroxybutyrate-C-14 for oxidation. The decreased C-14 uptake from the medium during incubation with leucine-U-C-14 and β-hydroxybutyrate could be ascribed to isotope dilution only if a large proportion of the C-14 in the medium after incubation pertained to labeled breakdown products of leucine-U-C-14 which diffused out of the diaphragm. Thus, it is not clear whether or not the decreased uptake and oxidation of C-14 from leucine-U-C-14 in the presence of β-hydroxybutyrate represents inhibition of leucine oxidation by the ketone body. The increased uptake of C-14 from leucine-U-C-14 and its accelerated oxidation to C-14-O_2 during incubation with 1 mM. octanoate probably represents accelerated oxidation of the amino acid or of its fragments.

Whether or not the stimulatory effect of octanoate on leucine oxidation is related to its inhibitory effect on protein synthesis is not clear. Octanoate has been reported to uncouple oxidative phosphorylation of rat liver mitochondria in vitro.[21] Octanoate (1 mM.) inhibited the incorporation of C-14 from lysine-U-C-14 into proteins of diaphragms incubated with insulin, while the uptake and oxidation of C-14 from lysine were not affected by octanoate (unpublished results).

Although we have demonstrated that palmitate, β-OH-butyrate and octanoate can inhibit protein synthesis in muscle under certain in vitro conditions, the physiological significance of these observations is not established. The concentration of palmitate (and the palmitate albumin ratios) used exceeded the range found in serum; the concentrations of β-OH-butyrate required to inhibit protein synthesis in vitro exceeded those seen in severe ketoacidosis. Octanoate was inhibitory at relatively low concentrations (0.5 mM.), however, octanoate is not a constituent of the mammalian FFA pool. The effectiveness of octanoate in inhibiting protein synthesis at low concentrations suggests that intermediate

chain length FFA may penetrate easier than other FFA into cellular compartments which are directly or indirectly involved in protein synthesis. Recent work indicates that octanoate infusions cause hypoglycemia in dogs, without increasing insulin secretion, probably by a direct inhibitory action on hepatic neoglucogenesis.[22]

Schonfeld and Kipnis studied the relationship between the extracellular and tissue fatty acid pools and the inhibition of glucose metabolism in "intact" diaphragms.[23] They found that the tissue fatty acid pool was not significantly influenced by increasing the palmitate concentration (or the albumin/palmitate ratio) of the incubation medium, while palmitate esterification by muscle increased with increasing medium FFA. They concluded that the tissue FFA pool is functionally heterogenous and that changes in glucose metabolism and insulin responsiveness correlate well with the tissue concentration of FFA but not with the extracellular concentration of palmitate.

The tissue FFA concentration in rat diaphragms is markedly influenced by several nutritional and hormonal factors. Increases of 200 to 400 per cent have been produced "in vivo" by fasting,[23,24] alloxan diabetes[23,25] and by the administration of epinephrine.[25] Epinephrine added in vitro increased the FFA content of isolated rat diaphragms severalfold.[25] The latter finding suggests that the epinephrine-induced increase in tissue FFA is a result of changes in the relative rates of tissue lipolysis/FFA utilization, rather than a reflection of the extracellular concentration of FFA.

Isolated rat diaphragms obtained from rats fasted for forty-eight hours incorporate less C-14 labeled amino acids into proteins than those from fed controls.[26] Epinephrine administered in vivo, or added in vitro to the incubation medium inhibits the incorporation of C-14 from amino acids into proteins in isolated rat diaphragms.[27] The results obtained in the present study with high concentrations of medium FFA suggest that protein synthesis in muscles may also be inhibited by increased intracellular FFA. It is tempting to speculate that intracellular FFA (or products of their metabolism) may have a regulatory influence on the rate of protein synthesis in muscle, or the rate of synthesis of certain muscle proteins. Such a mechanism would increase the supply of amino acids available for neoglucogenesis during stress or carbohydrate deprivation.

Manchester reported that insulin stimulates C-14 oxidation by rat diaphragms incubated with 1 mM. leucine-C-14.[15] In the present study, insulin did not stimulate C-14 oxidation from 0.05 mM. leucine-U-C-14. Similarly, during incubation with 0.05 mM. leucine-1-C-14, insulin failed to stimulate C-14 oxidation (unpublished results). The leucine concentration in the medium may be critical. As leucine in the medium is increased from 0.04 mM. to 1 mM. the C-14 fraction which is incorporated into proteins decreases from 40 to 10 per cent of the C-14 uptake.[13] When leucine in the medium is 0.002-0.06 mM., insulin stimulates its incorporation into proteins by 60 per cent; when leucine is 0.15 mM. the insulin effect is less than 20 per cent.[14] Since insulin increases the uptake of leucine from the medium (which implies accelerated penetration into the cell) and the rate of protein synthesis and since the intracellular amino acid pool is not homogenous,[10,11] it appears reasonable to postulate that at low extracellular leucine concentrations, the extra leucine that enters the cell under the influence of insulin is incorporated into proteins without becoming available for oxidation. At high extracellular leucine concentrations the protein synthetizing mechanism (although stimulated by insulin) may become saturated and more leucine becomes available for oxidation.

In the present study with 0.05 mM. leucine-U-C-14, insulin increased the uptake of C-14 from the medium, stimulated C-14 incorporation into proteins, did not affect C-14-O_2 production, and decreased the TCA soluble C-14 contained in the muscles. The latter finding suggests that either insulin accelerated protein synthesis more than it stimulated leucine transport, or that insulin promoted the incorporation of labeled breakdown products of leucine-U-C-14 into proteins[8] or lipids.

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THE ABSORPTION OF FATS STUDIED IN A CHILD WITH CHYLOTHORAX

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The way in which fat is absorbed in healthy people is still largely unknown. This lack of knowledge chiefly concerns the question whether complete hydrolysis of the dietary fat is necessary before it is absorbed, or whether absorption through the intestinal wall is possible without complete hydrolysis. In the past few decades this problem has been studied mainly by Verzár and McDougall (1, 2) and by Frazer (3–12), although in widely different ways.

The quintessence of Verzár's theory is: complete hydrolysis of fat in the intestine and synthesis of the fatty acids to triglycerides in the intestinal cells, *via* an intermediate stage of phospholipids. The main features of Frazer's theory are: hydrolysis of part of the fat into mono- and diglycerides and free fatty acids in the intestine, formation of an emulsion and absorption of tiny droplets of fat. The essential point of difference between these two theories is the degree of hydrolysis of the fat in the intestine. This problem has been approached in various ways.

The degree of hydrolysis was determined directly by examination of the intestinal contents after administration of fat by Mattson, Benedict, Martin, and Beck (13), Desnuelle and Constantin (14) and Kuhrt and other workers (15). Other attempts to determine the degree of hydrolysis using an indirect method of calculation, by comparison of chyle fat with dietary fat labelled with isotopes, were made by Borgström (16), Reiser, Bryson, Carr, and Kuiken (17, 18) and Bernhard, Wagner, and Ritzel (19). All these investigations led to the conclusion that there is a considerable hydrolysis of fat in the intestinal lumen. According to Frazer (10) about 40 per cent of the dietary fat is hydrolyzed partially, but Borgström and Reiser consider it highly probable that the hydrolysis, in any case into mono- and diglycerides, is practically complete. The latter authors assume that 25 to 45 per cent of the dietary fat is completely broken down into free fatty acids and glycerol; the remaining 55 to 75 per cent is, according to them completely, and according to Bernhard partially, hydrolyzed into monoglycerides.

There is, however, quite another aspect to this problem. Frazer (6–9), Bernhard (20), Borgström (21, 22), Bloom, Chaikoff, and Reinhardt (23) and Favarger (24) have shown that not all fatty acids are absorbed in the same manner.

According to Frazer the fatty acids of low molecular weight are absorbed in a different manner from those of high molecular weight. Frazer also deals separately with the unsaturated fatty acids, such as Δ^9-octadecanoic acid. As the dietary fats may be of widely varying composition, the determination of the individual absorption of all fatty acids, of which the dietary fat concerned is composed, is of great advantage, in contrast to the determination of the total absorption of fat or of the absorption of one particular labelled fatty acid.

The method of Boldingh (25, 26) enables such an analysis. A case of chylothorax in a child offered us a welcome opportunity to use this method in order to study the absorption of the separate fatty acids (27).

CLINICAL FEATURES

Chylothorax is a rare anomaly, arising when the pleural cavity of one or both lungs fills with chyle. The anomaly is caused by a lesion of a chyle vessel. The patient who gave us the opportunity of conducting this investigation was the third child of healthy parents; he was born on July 13, 1950. The other children were healthy. The mother's pregnancy was uneventful; parturition was normal. Directly following birth it was noticed that the patient had a swollen abdomen and a bilateral testicular hydrocele. When the boy was two months old, operation on a bilateral inguinal hernia (which had developed gradually) revealed that the swollen abdomen was due to chyloascites. Seven months later, a right-sided chylothorax was found in addition.

Cap Acid

The signs and symptoms of chylothorax and chyloascites alternated in predominance, without any operation being necessary, up to the age of nearly two years. Then he began to have attacks of dyspnea until he was admitted to the hospital, because of the severity of the dyspnea, at the age of 22 months.

On examination no other abnormalities were found apart from a barrel-shaped thorax and much fluid in the right pleural cavity. There was no fluid in the abdominal cavity. Feces, urine and blood were normal on morphological and chemical examination. Liver function and fat balance were also normal (the latter had been disturbed only for a short period during and after an infection). Although the patient's nutritional condition was not optimal, his growth during his affection and after recovery from it was always normal. On the strength of these data the patient was regarded as a healthy child with a purely anatomical abnormality. It may therefore be assumed that the chyle of this patient was also normal.

The right pleural cavity was drained weekly by aspiration during his eight months' stay in the Pediatrics Department. The secretion of chyle into the pleural cavity diminished during the last few weeks of this period and had ceased completely at the end of the eight months. So far relapses have remained absent and, at the time of writing, the child is in good condition.

The patient's chyle came up to the requirements of Baldridge and Lewis (28): 1) its color was that of milk; 2) a cream-like upper layer separated from the fluid at rest; 3) it was an emulsion of fine fat droplets; 4) its reaction was alkaline; 5) the fluid was sterile and not liable to decomposition; 6) the fat content was usually high, varying between 0.4 and 4.0 per cent; 7) the protein content was usually more than 3.0 per cent; 8) the fluid contained many lymphocytes. It appeared moreover that: the fat content of the chyle was dependent on the fat content of the diet; and, the fatty acid composition of the chyle fat was dependent on the fatty acid composition of the dietary fat. These relationships are characteristic of chyle and constitute proof that the fluid aspirated from the thoracic cavity was actually chyle. Results of laboratory examinations are shown in Table I.

TABLE I

Some characteristics of the patient's chyle

	Number of determinations	Range	Average
Cells (mainly mononuclears)	18	(900—6000)	3000/mm³.
Protein (Kjeldahl)	6	(3.37–4.57)	4.00 gm. %
Albumin	6	(1.79–2.54)	2.25 gm. %
($\alpha+\beta$)-Globulin	6	(0.14–0.60)	0.50 gm. %
γ-Globulin	6	(0.77–2.31)	1.25 gm. %
Cholesterol	25	(90–230)	130 mg. %
Cholesterol esters	25	(60–220)	65 mg. %
Potassium	6	(3.7–4.2)	4.0 mEq./L.
Sodium	6	(137–141)	140 mEq./L.
Calcium	6	(4.5–4.8)	4.7 mEq./L.
Chloride	6	(99–109)	103 mEq./L.
Phosphorus	6	(4.9–5.1)	5.0 mg. %

EXPERIMENTAL

Aim of the investigation

The aim of the investigation was to study the relationship between dietary fat and chyle fat, to arrive at conclusions on the absorption of fats in man, and to test these conclusions against the current theories on the mechanism of fat absorption.

Arrangement of the experiments

On the strength of animal experiments and based on Munk's investigations on a patient with a chylous fistula, it is to be expected that a great deal of the fat, under normal circumstances, is absorbed with the chyle. It is, therefore, possible to study the fat absorption, at any rate that via the lymph, by comparison of the composition of chyle fat with that of dietary fat. In contrast to former investigations, it is now possible to compare the composition of the chyle fat as a whole with that of the dietary fat, by means of separate quantitative determinations of all fatty acids. As not all intestinal chyle accumulates in the patient's thorax, absolute measurement of the absorption via the lymph was not possible.

The natural fats, which are mixed triglycerides being composed of widely varying proportions of the fatty acids, n-octanoic to n-docosanoic, were administered to the patient in such a way that each time certain particular fatty acids could be studied. When the patient was given butterfat, the emphasis fell on the short-chain fatty acids, n-tetranoic, n-hexanoic, and n-octanoic; when given margarine and coconut oil, n-octanoic, n-decanoic, and n-dodecanoic acid were preponderant. A synthetic fat, "capryl olive oil," was administered to study the behavior of n-octanoic acid more closely; cacao-butter was given to examine that of n-hexadecanoic and n-octadecanoic acid; and finally a synthetic fat with hydrogenated herring oil was given to investigate the absorption of n-eicosanoic and n-docosanoic acid.

The next step was to study the absorption via the lymph of some *unsaturated fatty acids*. By feeding sunflower oil and linseed oil the absorption of $\Delta^{9,12}$-octadecadienoic acid and $\Delta^{9,12,15}$-octadecatrienoic acid was investigated, while the behavior of Δ^{13}-docosenoic acid was studied by the administration of rapeseed oil. Comparison of the lymphatic absorption of olive oil and cláidinized olive oil enables us to investigate the absorption of Δ^{9}-octadecenoic acid (both cis- and trans-isomers).

As the lymph was aspirated only once a week, the possibility could not be excluded that some fatty acids might be absorbed from the thoracic cavity to a greater extent than others. This is, however, unlikely because the fatty acids are almost completely present as fat in the lymph. A differentiation is therefore impossible here and a specific absorption can therefore not be expected.

It is possible that part of the fat is absorbed via the portal system and the liver. This was not investigated in the case concerned. The possibility should not be overlooked that some fat escapes absorption and is excreted in the feces. In order to keep a check on this the fat balance was always determined by the method of van de Kamer (29).

The patient was kept on a diet with constant amounts of fats, proteins, and carbohydrates during most of the experiments. The daily diet consisted of:

200 ml. protein milk [1]	75 gm. green vegetable
20 gm. rice flour	25 gm. lean meat
20 gm. sugar	16 gm. fat
400 ml. water	75 gm. banana
50 gm. potato	50 ml. orange juice
100 ml. tomato juice	

Vitamins A, D, B-complex [2] and C were added to this diet. Stabilized ferrous chloride was also given per os. The amount of fat was 28 gm. per day; exceptions were made during some periods in which larger or smaller amounts were given. It was necessary during the last two months to increase the amounts of protein and carbohydrate in order to meet the increasing caloric demands.

The type of fat in the diet was changed every week, or group of weeks, by giving a protein milk and fat containing different kinds of glycerides, such as coconut oil or olive oil instead of butterfat.

The chyle was collected by pleural puncture at the end of each week, and subsequently the pleural cavity was examined radiologically for the presence of chylous residues. Because of the possibility that a slight residue may influence the composition of the chyle during the following dietary period, a diet containing one kind of fat was usually given for two successive weeks. The composition of the chyle fat during the second week was considered the most representative.

Methods

The chemical investigation was carried out as follows: All saturated fatty acids with the exception of n-tetranoic acid were quantitatively determined separately. Of the unsaturated fatty acids, only the poly-unsaturated ones could be determined individually by measurement of the absorption spectrum in ultraviolet light after alkaline isomerization. It was impossible to determine the mono-unsaturated fatty acids directly, either separately or *in toto*. The sum of the unsaturated fatty acids was computed by subtracting the total of the saturated fatty acids from the total of all fatty acids. The iodine number also lent support to this calculation. With these restrictions the fatty acid composition of each dietary fat could be compared with that of the corresponding chyle fat. The extraction and analyses of the fat from chyle, feces and blood were carried out by the methods of van de Kamer and his colleagues (30), using the analytical method of Boldingh (25, 26). A mixture of the analytically pure saturated fatty acids, n-hexanoic to n-docosanoic, which contains of each fatty acid 2 to 8 mg., can be analyzed with this method so that each acid is estimated with an accuracy of about 5 per cent. The minimum amount which can be determined with this accuracy is 0.5 to 1.0 mg., depending on the molecular weight.

RESULTS

The chyle fat during a low-fat diet

The chyle fat does not give an accurate impression of the intestinal lymph fat because the chyle consists of a mixture of intestinal and non-intestinal lymph. This should be kept in mind when a comparison is made of the fatty acid composition of chyle fat with that of dietary fat. In other words, if the chyle fats are to be compared with the corresponding dietary fats, a correction must be made for the admixture with non-intestinal lymph fat. The composition of the fat in the non-intestinal lymph is unknown, but it may be assumed that chyle fat collected during a low-fat period is similar to non-intestinal lymph fat, because the fat in the chyle during such a low-fat diet can not be of intestinal origin. The correction factor was determined by analyzing the chyle fat of four low-fat periods. Both the fatty acid composition and the fat content of this chyle were practically constant in all the periods. From this it follows that the composition of the "corrected chyle fat" can be calculated by subtracting the non-intestinal fat from the chyle fat as found by analysis. Then the fatty acid pattern of the "corrected chyle fat" can be compared directly with that of the dietary fat.

The patient was on a low-fat diet for four successive periods of a week each, the amounts of

[1] The protein milk was prepared by Nutricia, Ltd., of Zoetermeer, The Netherlands, and consisted of a base of buttermilk in which 5.5 gm. per 100 ml. butterfat, coconut oil, olive oil, etc. were emulsified without the addition of an emulsifier. In some periods the fat was emulsified in skimmed milk.

[2] One tablet of Vitamin B-complex daily was administered, containing: thiamin 5 mg., riboflavin 2 mg., nicotinic acid amide 20 mg., adermin 2 mg., and calcium pantothenate 3 mg.

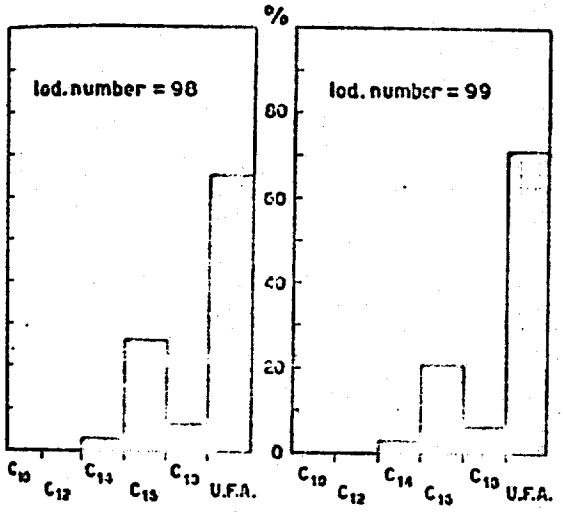

FIG. 1. LEFT: COMPOSITION OF THE PATIENT'S CHYLE FAT DURING A LOW-FAT DIET (LESS THAN 1 GM. FAT DAILY FOR FOUR WEEKS). RIGHT: COMPOSITION OF THE BLOOD FAT OF THE FASTING PATIENT (DURING A DIET CONTAINING RAPESEED OIL)

Solid columns = saturated fatty acids.
Striped columns = unsaturated fatty acids (U.F.A.)

fat given being less than 1 gm. daily. The chyle, which was of a milky white color during a regimen with normal quantities of fat (28 gm. daily), became transparent during the low-fat diet; in appearance it resembled blood plasma. The fat content of the chyle fell markedly, from 3.7 to about 0.6 gm. per cent.

Figure 1 shows the composition of the chyle fat during a low-fat regimen. For purposes of comparison the fatty acid pattern of the blood fat of the fasting patient during another dietary period is also given. There is a striking resemblance between the two fatty acid patterns (31). This does not imply that the forms in which these fatty acids occur (triglycerides, phospholipids, and cholesterol esters) are also similar. Table II shows that during the first period of the low-fat diet there was still an admixture with "high-fat" chyle from the preceding margarine period. In the three following periods, however, the chyle was characterized by a constant low fat content and a constant fatty acid composition. The iodine number of this chyle fat was as high as that of the fat of the blood serum of the fasting patient (see Figure 1).

Table II also shows the composition of the chyle fat of a patient with chyloascites from the Hospital for Sick Children, Great Ormond Street, London. The chyle of this nine-month-old patient was collected during a low-fat diet and was put at our disposal by Drs. W. W. Payne and A. Norman. It appears from these data that the fatty acid pattern of the chyle from their patient shows a resemblance to that of the "low-fat" chyle from ours (see Table II).

The chyle fat during a diet containing butterfat, coconut oil or margarine

The dietary fats and the chyle fats were analyzed in the usual manner, while the n-tetranoic acid content was determined separately by the method of Fairbairn and Harpur (32). The results are shown in Figure 2. The figures on the left show the percentage composition of the dietary fat in comparison with the "corrected" composition of the chyle fat. Those on the right give curves of the lymphatic absorption coefficient of each fatty acid of the dietary fat. These coefficients were cal-

TABLE II

The per cent of fatty acids in the fat from the chylothorax and chyloascites of our patient and the London patient, respectively

Fatty acid	Chylothorax patient: Margarine diet 28 gm. per day	Chylothorax patient: Low-fat diet I	Low-fat diet II	Low-fat diet III	Low-fat diet IV	Chyloascites patient: Low-fat diet
n-Decanoic (C_{10})	1	—	—	—	—	—
n-Dodecanoic (C_{12})	10	7	<1	1	0	1
n-Tetradecanoic (C_{14})	9	7	3	2	2	2
n-Hexadecanoic (C_{16})	19	21	26	26	28	25
n-Octadecanoic (C_{18})	6	6	6	6	7	10
n-Eicosanoic (C_{20})	—	—	—	—	—	1
Total unsaturated fatty acids	55	59	64	66	63	61
Iodine number	64	77	93	93	89	58
Total fat content of chyle, in per cent	3.70	1.22	0.54	0.52	0.58	0.21
Coefficient of fat absorption, in per cent	96	—	—	—	—	—

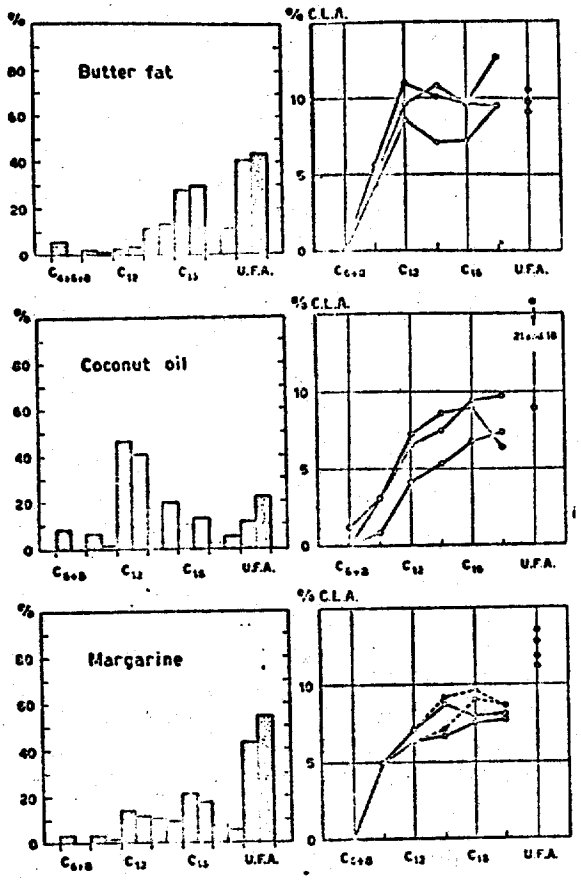

FIG. 2. LEFT: COMPOSITION OF DIETARY FAT AND OF CHYLE FAT (CORRECTED) DURING DIETS CONTAINING RESPECTIVELY BUTTER FAT, COCONUT OIL AND MARGARINE. RIGHT: COEFFICIENTS OF LYMPHATIC ABSORPTION (C.L.A.) OF THE SATURATED AND UNSATURATED FATTY ACIDS

Solid columns = saturated fatty acids in dietary fat.
Open columns = saturated fatty acids in chyle fat.
Striped columns = unsaturated fatty acids in dietary fat.
Stippled columns = unsaturated fatty acids in chyle fat.
O———O = "corrected values."
o - - - - - - - - o = "uncorrected values."

culated by determining the percentage of each fatty acid of the dietary fat which was recovered in the chyle. This percentage is called the "coefficient of lymphatic absorption" (C.L.A.) of the fatty acid. We are, of course, quite aware that these absorption coefficients do not indicate the total absorption of a given fatty acid via the lymph because the drainage of chyle from the patient was only partial. Only about 10 per cent of the fat from the food is recovered as chyle fat. This quantity, which was about 15 gm. chyle fat per diet period, is large enough for the calculation of the C.L.A. of each fatty acid as follows:

$$\text{C.L.A.} = \frac{\text{mg. fatty acid in chyle}}{\text{mg. fatty acid in food}} \times 100$$

These figures, therefore, merely indicate how much fatty acid of the dietary fat was found in the aspirated chyle, which was only part of the total chyle. The C.L.A.'s, however, permit comparison of the various fatty acids with each other with respect to their lymphatic absorption.

The diagrams demonstrate a resemblance between the composition of the chyle fat and the dietary fat. This holds true for butterfat, coconut oil, and margarine. On closer consideration, however, certain differences become clearly manifest when the various C.L.A.'s are taken into account (see the graph on the right). n-Tetranoic, n-hexanoic and n-octanoic acid are completely absent in the chyle; the C.L.A. of n-decanoic acid is 3 to 5 per cent; of n-dodecanoic acid 7 to 10 per cent; of n-tetradecanoic acid somewhat more, just as for n-hexadecanoic and n-octadecanoic acid. In this way it is demonstrated that the C.L.A. increases when the chain length of the fatty acids increases. The unsaturated fatty acids are absorbed as well as or somewhat better than the saturated n-tetradecanoic, n-hexadecanoic, and n-octadecanoic acids.

The chyle fat during a diet containing "capryl olive oil"

As it was a very striking feature that the short-chain fatty acids, n-tetranoic, n-hexanoic, and n-octanoic, were not found in the chyle when the patient was given margarine, butter, or coconut oil, it was of importance to verify this observation by administering one of these fatty acids in a greater amount in the form of triglyceride. The following data gathered from the literature are given for purposes of comparison. Kiyasu, Bloom, and Chaikoff (33) administered to rats 1-C^{14}-labelled n-decanoic acid dissolved in maize oil. The lymphatic absorption of this free fatty acid was only 7 to 19 per cent. Frazer used tributyrine (the triglyceride of n-tetranoic acid) which was not found in the chyle after administration to rats. One should, however, be cautious in drawing con-

clusions from these experiments because a free fatty acid was used in the first experiment, which is an unphysiological procedure. In the second experiment a single triglyceride was employed, not to be compared with the natural fats which are composed of mixed triglycerides. Moreover, in contrast to the other long chain fatty acids, n-tetranoic acid is very readily soluble in water. Apart from these objections the experiments show at most that some short-chain fatty acids are not absorbed in the chyle or absorbed only to a very slight degree. We observed the same phenomenon in the absorption of mixed triglycerides in which the short-chain fatty acids, n-tetranoic, n-hexanoic and n-octanoic acid, from margarine, butterfat, and coconut-oil were also absent in the chyle. At the same time, however, the long-chain fatty acids were demonstrated in the chyle. This can only be explained by hydrolysis of the dietary fat.

In the experiment described below, a fat was chosen which would clearly show whether or not hydrolysis occurred. Use was made of "capryl olive oil," a fat containing 46 per cent n-octanoic acid. This was prepared by subjecting a mixture of equal parts of olive oil and tricapryline, the triglyceride of n-octanoic acid, to alkaline re-esterification. In this way a mixed triglyceride was formed in which each molecule contained at least one n-octanoic acid group.[3] This "capryl olive oil" was given to the patient in the usual quantities (28 gm. daily) for one week. The fat balance was normal during this period (absorption coefficient 98 per cent). The fat content of the chyle also retained a normal value (2.1 per cent). This proves that the fat absorption in general, as well as the lymphatic absorption in particular, was normal. For these reasons this experiment with a synthetic fat may be considered a physiological one.

Figure 3 shows that the chyle fat contained only 4 per cent n-octanoic acid in contrast to the dietary fat which contained 46 per cent of this acid. The C.L.A. of n-octanoic acid is only 0.70 per cent, while that of Δ^9-octadecanoic acid is 8.6 per cent. These experiments therefore confirm the findings

[3] The composition of the "capryl olive oil" was calculated as: 27.5 mol. per cent tricapryline; 44.3 mol. per cent "dicapryl olive oil;" 23.9 mol. per cent "monocapryl olive oil;" and 4.3 mol. per cent olive oil.

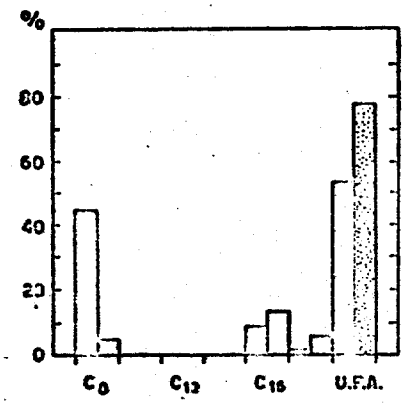

FIG. 3. COMPOSITION OF DIETARY FAT AND OF CHYLE FAT (CORRECTED) DURING A DIET CONTAINING "CAPRYL OLIVE OIL"

Solid columns = saturated fatty acids in dietary fat.
Open columns = saturated fatty acids in chyle fat.
Striped columns = unsaturated fatty acids in dietary fat.
Stippled columns = unsaturated fatty acids in chyle fat.

when butterfat, coconut oil, and margarine are used.

As n-octanoic acid was found in the chyle in only slight amounts, while the "capryl olive oil" was well absorbed (the fat balance was normal), it seemed acceptable that this fatty acid was absorbed via the portal vein or converted in the intestinal wall (< 12.5 mg. per cent octanoic acid). Whether n-octanoic acid could be demonstrated in the blood was therefore investigated. Three and one-half hours after an oral dose of 28 gm. "capryl olive oil" (*i.e.*, the whole amount for one day) venepuncture was carried out and the blood fat analyzed. n-Octanoic acid could not be demonstrated in it, and it may therefore be concluded that n-octanoic acid does not pass the liver as such but is converted in the intestine or liver.

The chyle fat during a diet containing cacao-butter and hydrogenated herring oil with peanut oil

In order to study in more detail the lymphatic absorption of the saturated fatty acids, n-hexadecanoic, n-octadecanoic, n-eicosanoic and n-docosanoic, experiments were carried out with cacao-butter, a fat containing 25 per cent n-hexadecanoic and 35 per cent n-octadecanoic acid, and with a hydrogenated herring oil (re-esterified with peanut oil so that the melting point was 42°C.), a fat containing 8 per cent n-eicosanoic and 8 per cent

n-docosanoic acid. No conclusions can be drawn regarding the absorption of n-hexadecanoic and n-octadecanoic acid because the cacao-butter diet was preceded by a period during which the patient suffered from enteritis. Although he seemed clinically cured, there was still present a steatorrhea (absorption coefficient = 80 per cent), the fat of which contained much hexadecanoic and n-octadecanoic acid. The low coefficient of absorption of cacao-butter might be caused by the high percentage of stearic acid in this fat, *viz.* 35 per cent. This is in accordance with the results of Bloom, Chaikoff, and Reinhardt (23) who found that only 36 to 54 per cent of stearic acid, as free fatty acid dissolved in corn oil, is absorbed by the rat, in contrast to at least 80 per cent of shorter chain fatty acids.

It can only be deduced that n-eicosanoic (C_{20}) and n-docosanoic (C_{22}) acids are absorbed through the lymph. It was, however, impossible to calculate the degree of this absorption because the chyle production had fallen markedly during this period, owing to the patient's spontaneous recovery.

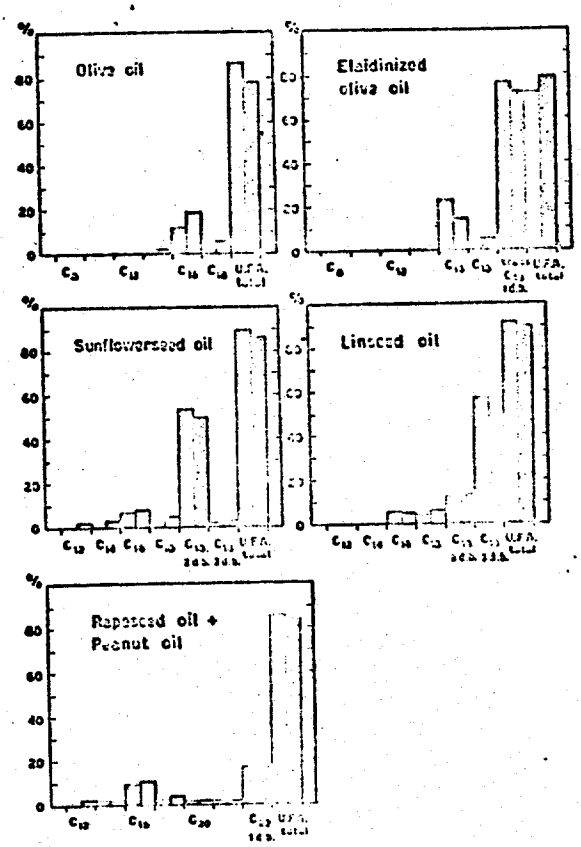

FIG. 4. COMPOSITION OF DIETARY FAT AND OF CHYLE FAT (CORRECTED) DURING DIETS CONTAINING FATS WITH UNSATURATED FATTY ACIDS

Solid columns = saturated fatty acids in dietary fat.
Open columns = saturated fatty acids in chyle fat.
Striped columns = unsaturated fatty acids in dietary fat.
Stippled columns = unsaturated fatty acids in chyle fat.

The chyle fat during diets containing fats with unsaturated fatty acids

In order to study more closely the absorption of various unsaturated fatty acids, Δ^9-octadecenoic acid (cis and trans-isomers), $\Delta^{9,12}$-octadecadienoic acid, $\Delta^{9,12,15}$-octadecatrienoic acid, and Δ^{13}-docosenoic acid, experiments were carried out with olive oil (containing cis-Δ^9-octadecenoic acid), elaidinized olive oil (containing trans-Δ^9-octadecenoic acid), sunflower seed oil (containing $\Delta^{9,12}$-octadecadienoic acid and $\Delta^{9,12,15}$-octadecatrienoic acid), linseed oil (*idem*) and rapeseed oil + peanut oil (containing Δ^{13}-docosenoic acid). The results are represented in Figure 4. In all cases there was a great degree of resemblance between the composition of chyle fat and dietary fat.

Summary

The results of the entire investigation are comprised in Figure 5. The composition of the chyle fat remained practically unchanged in successive periods when the same dietary fat was given. On the other hand, when a change was made in the dietary fat a simultaneous change in the chyle fat was observed. The similarities and differences between the dietary fat and the corresponding chyle fat are also evident in the Figure.

DISCUSSION

Experiments have indicated that the fatty acid pattern of the chyle fat differs from that of the dietary fat.

Of the *saturated fatty acids*, n-tetranoic and n-hexanoic are not present in the lymph, while n-octanoic is found only to a very slight degree, even if this fatty acid is present in large amounts in the dietary fat. n-Decanoic acid is found in the lymph but its absorption is less than that of n-dodecanoic, n-tetradecanoic, n-hexadecanoic, and

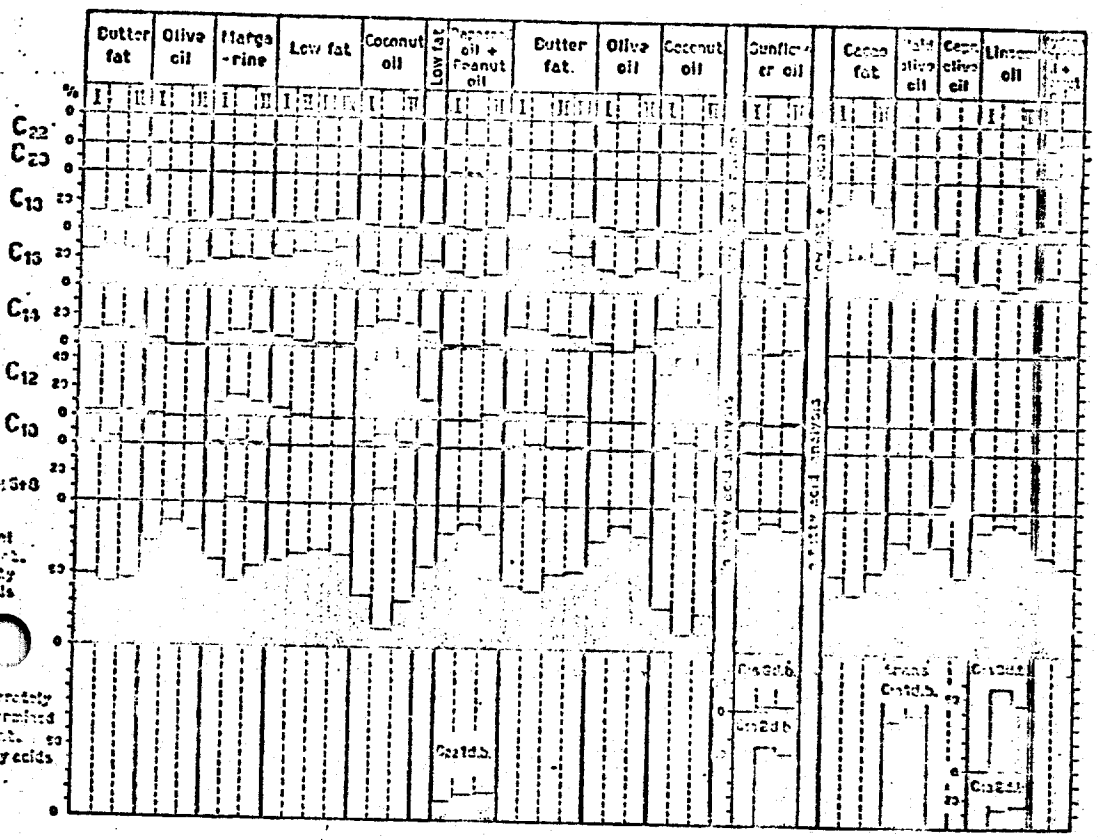

FIG. 5. COMPARISON OF THE FATTY ACIDS OF THE DIETARY FAT WITH THE FATTY ACIDS OF THE CHYLE, UNCORRECTED VALUES, REPRESENTED IN CHRONOLOGICAL ORDER DURING ALL DIETS OF THE WHOLE PERIOD OF OBSERVATION

Solid columns = fatty acids of diet.
Stippled columns = fatty acids of chyle.

-octadecanoic. The four last-mentioned fatty acids are absorbed practically to the same extent. This can be deduced from Figure 6 which shows the coefficients of lymphatic absorption (C.L.A.) of the saturated fatty acids of various fats. The lines run closely parallel in the same direction which proves clearly that the C.L.A.'s of n-hexanoic, n-octanoic, n-decanoic, and n-dodecanoic acid increase as the carbon chains become longer.

In order to compare the C.L.A.'s of the fatty acids in the various periods, it is necessary to eliminate the differences in the production of chyle during these periods (see Figure 7). Although some objections may be raised to this procedure, the fatty acids found in the chyle were recalculated for a standard chyle production of 500 ml. per week. This seems to be justifiable since the fat content of the chyle was independent of the quantity of chyle produced per week and also because the amount of chyle was independent of the nature of the dietary fat. The diminution of the quantities of chyle aspirated weekly was due to the patient's spontaneous recovery.

The various *unsaturated fatty acids* were absorbed well. The C.L.A.'s of these fatty acids are of the same order as those of the saturated fatty acids, n-tetradecanoic, n-hexadecanoic, and n-octadecanoic.

It follows from the difference in lymphatic absorption of the saturated fatty acids, n-octanoic, n-decanoic, and n-dodecanoic, that the dietary fat must be hydrolyzed at least partially during absorption. Otherwise, if dietary fat was absorbed without hydrolysis, there would be no difference in the absorption coefficient of the three mentioned fatty acids.

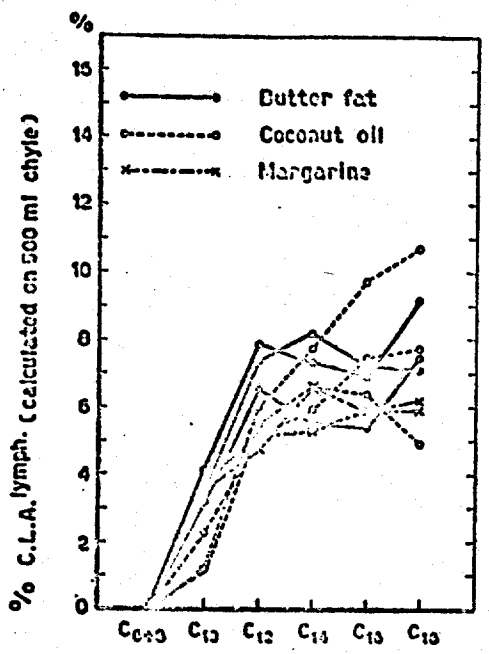

FIG. 6. COEFFICIENTS OF LYMPHATIC ABSORPTION (C.L.A.) OF VARIOUS DIETARY FATS CALCULATED FOR 500 ML. CHYLE

Thus the results of the experiments with butter, margarine, and coconut oil may be explained by hydrolysis of *part* of the dietary fat. The other part of it might pass through the intestinal wall as triglyceride and be absorbed with the chyle. In the experiment with "capryl olive oil" almost every molecule of fat must have been hydrolyzed, as Figure 5 shows that the C.L.A. of n-octanoic acid is only 0.7 per cent, while the coefficient for the longer chain fatty acids of this fat amounts to 8.6 per cent.

The result of the very low lymphatic absorption of n-octanoic acid is also clearly manifest on comparison of the composition of dietary fat. The dietary fat contained 46 per cent n-octanoic acid while only 4 per cent of this acid was recovered in the chyle fat. The practical absence of n-octanoic acid in the chyle fat can only be explained by the assumption of a hydrolytic breakdown of almost every molecule of fat because each fat molecule contains an n-octanoic acid group. This hydrolysis, however, is not conceivable when the fat is present in the intestine in the form of tiny globules as the lipase cannot act on each fat molecule under these circumstances.

On the strength of the above considerations ("capryl olive oil"), the assumption that practically every triglyceride molecule undergoes hydrolysis does not imply that each fat molecule is hydrolyzed completely (1–2); it is quite possible that only one or two molecules of fatty acid are split off, so that mono- and diglycerides are formed.

It may be that the intestine possesses the ability of dealing with a fat, molecule by molecule. Hence the following differentiation might be possible:

1. Certain fats might be absorbed almost without hydrolysis, *e.g.*, the greater part of the multiedly unsaturated fats, olive oil, sunflower seed oil, linseed oil, and rapeseed oil.

2. Other fats must first be hydrolyzed completely or partially, *viz.* the fats containing short-chain fatty acids. After resynthesis, transport to the chyle follows, but the liberated short-chain

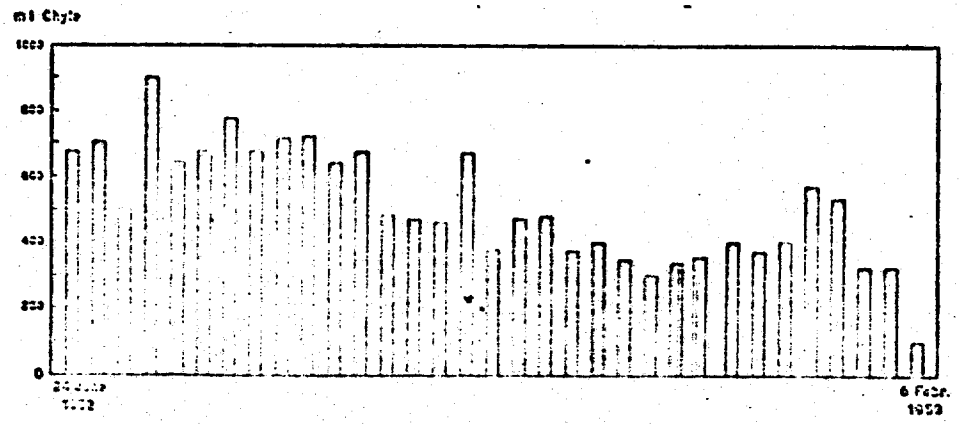

FIG. 7. THE QUANTITY OF CHYLE ASPIRATED DURING THE SUCCESSIVE PERIODS OF THE INVESTIGATION

fatty acids are converted in the liver or in the intestinal cells. This may be the case in the absorption of "capryl olive oil," butter, margarine, and coconut oil.

This hypothesis concerning the absorption of fats implies that the droplets of the fat emulsion in the intestine (3–12) disintegrate into molecules during absorption, with, if necessary, a selection of the fatty acids of which these molecules are built up. The fat droplets in the chyle are consequently of a different composition from the fat droplets of the dietary fat in the intestinal lumen, unless we are dealing with the absorption of the unsaturated fats.

A selective splitting off of special fatty acids from the *fat droplets* is hardly probable, the more so because these are surrounded by a layer of mono- and diglycerides (3–12). Frazer's view that the fatty acids n-tetranoic to n-decanoic are removed selectively from the fat droplets after hydrolysis, owing to their solubility in water, is debatable, because only the solubility of n-tetranoic acid in water is very high, while those of n-hexanoic, n-octanoic and n-decanoic acid decrease successively with rapidity. The above selection may take place in the intestinal contents or in the intestinal cells.

CONCLUSIONS

It may be stated that the nature of a dietary fat decides whether or not it is hydrolyzed in the intestinal lumen and subsequently partially resynthesized to chyle fat in the intestinal wall. If there is a hydrolysis followed by resynthesis, these processes are such that the degree of lymphatic absorption is different for each fatty acid. This means that the absorption mechanism is accompanied by considerable selective capacity. Selection of triglycerides or fatty acids, however, is only possible if the fat droplets, each actually consisting of millions of molecules, disintegrate.

On the strength of this idea it is conceivable that some dietary fats (olive oil, sunflower seed oil, etc.) pass more or less unchanged into the chyle, and that in the case of other fats (butter, coconut oil, margarine, etc.) the fatty acid pattern of the chyle fat differs considerably from that of the dietary fat.

SUMMARY

The absorption of fat via the lymph was studied in an otherwise healthy child with chylothorax by administering various dietary fats during two-week periods and comparing the fatty acid composition of dietary fat and chyle fat.

It appeared that the various fatty acids were not absorbed to the same extent. n-Hexanoic, n-octanoic and n-decanoic acid were not found, or found to only a slight degree, in the chyle. This was confirmed with an especially prepared fat, "capryl olive oil," containing 46 per cent n-octanoic acid.

The following hypothesis was evolved, based on the experiments described: Certain fats may be absorbed almost without hydrolysis, *e.g.*, the greater part of the markedly unsaturated fatty acids (such as olive oil, sunflower seed oil, and rapeseed oil). Other fats must first be hydrolyzed completely or partially, *viz.*, the fats containing short-chain fatty acids, such as butter, coconut oil, and "capryl olive oil." In the latter case this hypothesis cannot be reconciled with Frazer's theory of particulate absorption.

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THE RELATIVE RATE OF PENETRATION OF THE LOWER SATURATED MONOCARBOXYLIC ACIDS INTO MAMMALIAN ERYTHROCYTES

53

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FOUR FIGURES

Since the work of Overton (1895, 1899, 1902) the idea has been widely accepted that compounds soluble in lipid solvents penetrate cells by reason of their solubility in the lipids of the cell surface. A number of investigators (Loeb, Harvey, Crozier, Jacobs, Bodansky, Collander and Bärlund, Höber and Orskov, Dziemian) have tested this theory and, in general, the relative rate of penetration of the lipid soluble compound has been found to parallel its distribution in an oil-water mixture. These investigators used various methods to measure penetration of lipid soluble compounds, not all of which are equally reliable. Thus Loeb ('09, '13) using Arbacia eggs, studied the rate of fertilization membrane formation on exposure to the lower fatty acids. He found that as the fatty acid chain was lengthened, the membranes formed more rapidly, and considered his observations to support the Overton theory. However, in the absence of information showing that penetration must occur before membrane formation can take place, the Loeb results are best considered inconclusive support for the theory. Harvey ('14) and Crozier ('16) studied the penetration of acids into tissues of two invertebrate forms

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Comp. Acid.

which contained natural indicators. A color change in the indicator was taken as evidence of penetration of the acid. Both investigators found valeric or isovaleric acid to penetrate more rapidly than lower acids.

The studies of Jacobs ('26), Bodansky ('28), Höber and Orskov ('33) and Dziemian ('39) were made on erythrocytes using hemolysis as a criterion of penetration. This method has many advantages when used critically but, as will be shown by this report, cannot be accepted as a measure of penetration of compounds which enter cells more rapidly than water, without reservation. Of this group of hemolysis studies only those of Jacobs and Bodansky were concerned chiefly with the penetration of fatty acids into erythrocytes. Jacobs, using the ammonium salts of the fatty acids from formic to valeric, was able to show an increasing rate of penetration for this series. This is in agreement with the Overton theory. Bodansky studied the hemolytic effects of the fatty acids themselves. However, he neglected to distinguish between hemolysis wrought by injury and that produced by penetration of the acid and water and it is difficult to evaluate his results. The work of Collander and Bärlund ('33) dealt with the penetration of lipid soluble compounds in the cells of the large alga, Chara. They determined the amount of a compound which had entered the cell by chemical analysis and found for the most part agreement between the rate of penetration and the lipid-solubility of the compound. They did not study fatty acid penetration.

The present study has attempted to measure the relative rate of penetration of the lower fatty acids into mamalian erythrocytes by a new method. This method is essentially chemical and depends upon the fact that oxyhemoglobin when placed in an environment containing an increased hydrogen ion concentration loses some of its oxygen. This loss of oxygen may be determined spectrophotometrically. Under experimental conditions the rate at which oxyhemoglobin is converted into reduced hemoglobin in the intact erythrocyte in a fatty acid environment may be used as a measure of the rate

of fatty acid penetration into the erythrocyte. By the use of this method the rate at which the lower fatty acids, from formic through caprylic, penetrated beef and human erythrocyte was measured. It will be shown that the penetration rate was slowest for formic acid, increased to valeric acid and then again decreased in such a manner that heptylic and caprylic acids were found to penetrate more slowly than butyric acid. Lytic hemolysis studies were done with the Parpart densimeter ('35) and it was found that the results obtained in this way differed from those penetration data obtained by the spectrophotometric method.

METHODS

Since the work of Hüfner (1879) the absorption spectrum of oxyhemoglobin has been known and with instrumental improvement has been measured with increased accuracy down to the present time (Hari, '17; Drabkin, '35; Rubinstein and Ravikovich, '46). In a dilute suspension of erythrocytes oxyhemoglobin may be shown to change its absorption characteristics, as measured spectrophotometrically, by lowering the oxygen tension of the environment or by increasing the hydrogen ion concentration in the interior of the cells. This latter finding by Barcroft and Murray ('22) provides a means of measuring by the use of a spectrophotometer the relative amount of acid which has penetrated a suspension of erythrocytes. Hartridge and Roughton in a series of studies ('23, '25) have shown that under their conditions of measurement the dissociation of oxyhemoglobin at lower pH values is nearly complete within 1/50 of a second. This information makes feasible the measurement of the rate of penetration of acid compounds into erythrocytes provided such penetration occurs more slowly than the limiting dissociation time of oxyhemoglobin.

A device was made for the rapid mixing of red cell suspension and acid solutions. A drawing of this appears in figure 1. It consists essentially of two reservoirs, 6 and 7

which empty through stopcocks, 8 and 9 either through the exit tubes 10 and 11 or through the master stopcock, 12, into a mixing chamber, 13. The mixing chamber, shown in the inset, is so constructed that complete mixing occurs within it and a homogeneous mixture runs into the observation tube, 14. The entire device was made of pyrex glass. For experi-

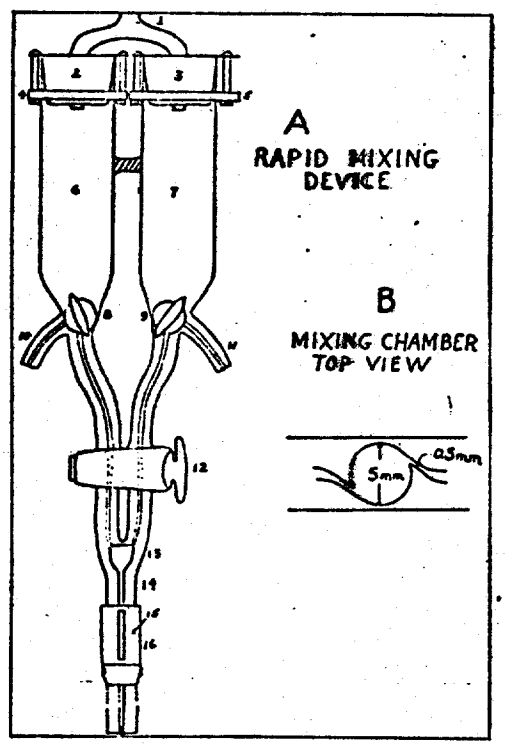

Fig. 1 Sketch of rapid-mixing device.

mental use the rapid mixing device was mounted above a Coleman, model 10S, spectrophotometer in such a manner that the observation tube passed vertically through the cuvette holder of the spectrophotometer. The monochromatic beam of light from the spectrophotometer then passed through the lumen of the observation tube. In order to focus the monochromatic beam to insure its passage through the lumen of

the tube, a glass collar (16 in fig. 1) was made of optically flat glass sealed together with DeKhotinsky cement and held in place on the observation tube by a piece of rubber tubing which readily permitted changing its position as experiment required. A piece of thin brass shim containing a longitudinal slit the same width as the lumen of the observation tube and the same length as the beam of light from the spectrophotometer was inserted inside the glass collar on the exit side of the light beam. When in use the glass collar was filled with glycerol which has approximately the same index of refraction as does pyrex glass.

When a red cell suspension is placed in one reservoir of the rapid mixing device and a dilute solution of a lower fatty acid in the other, the two liquids will come together in the mixing chamber when the large stopcock is opened. From here they pass through the observation tube, traveling enroute through the monochromatic beam of the spectrophotometer. It is apparent that the time which has elapsed from initial contact in the mixing chamber until the mixture passes through the center of the monochromatic beam represents the reaction time or the time in which the acid has had opportunity to enter the cell and bring about a dissociation of the oxyhemoglobin to a new equilibrium. Experimentally a measurement is made of the amount of light transmitted through the observation tube when the mixture is moving, i.e., a flow reading, and when it is stopped, i.e., a static reading. The difference between the two readings is taken as a relative measure of the acid which penetrated the cells in a given reaction time. The reaction time was varied by changing the mixing distance, i.e., the distance from the mixing chamber to the center of the monochromatic beam of the spectrophotometer, or by changing the speed of flow of the liquids leaving the reservoirs. The latter object was accomplished by fastening two stoppers (2 and 3 in fig. 1) to the tops of the reservoirs and connecting the reservoirs to a compressed air source. By calibration the reaction times for a series of mixing distances

and pressures were determined. The following equations were used in the calculation of a given reaction time:

$$\text{L.F. (cm/sec)} = \frac{\text{V(cm}^3\text{/sec)}}{\text{A(cm}^2\text{)}} \qquad (1)$$

This equation states that the linear flow (L.F.) is equal to the volume (V) of liquid in milliliters per second passing through the observation tube divided by the area (A) of the lumen of the observation tube.

$$\text{R.T. (sec)} = \frac{\text{D(cm)}}{\text{L.F. (cm/sec)}} \qquad (2)$$

This second equation states that the reaction time (R.T.) measured in seconds is equal to the mixing distance (D) in centimeters divided by the L.F. obtained from the first equation.

Beef blood was obtained from one of the local slaughter houses and human blood was secured from laboratory personnel. Both types of blood were citrated at the time of collection to prevent clotting and were subsequently strained through sterile gauze to remove any clots which may have formed. Whole blood was used in the preparation of the red cell suspensions in concentrations of 1 to 1.5% blood in 1% NaCl solutions. Because of the initial acidity of the saline solutions a small amount of NaOH was added to bring the pH of the red cell suspension to approximately 7.4. Buffer solutions were not used because the method depended upon changing the pH of a red cell suspension from about 7.4 to 4.2. Further, one wished to study the penetration of the acid molecule and not the acid anion. The lower members of the saturated, monocarboxylic acid series from formic through caprylic acid were studied. Stock molar solutions of formic, acetic, propionic and butyric acids were prepared and appropriate dilutions made from these. Because of the relative insolubility of valeric, caproic, heptylic and caprylic acids, dilutions were prepared directly from the concentrated acid. Dilute acid solutions were prepared and used on the same day. Table 1 lists the acid concentrations used with the ap-

TABLE 1

ACID	MOLAR CONC. OF ACID IN DILUENT	ESTIMATED CONC. OF ACID IN FINAL MIXTURE	AVER. pH VALUE OF MIXTURE	RATIO OF SALT/ACID AT AVER. pH	SURFACE [1] TENSION DYNES/CM	MR_D [2]	DISTRIBUTION [3] RATIO
Formic	0.006	0.003	4.34	4.68/1	72.51	8.55	0.005 (0.1 N acid)
Acetic	0.008	0.004	4.18	0.282/1	70.78	12.98	0.03 (0.1 N acid)
Propionic	0.01	0.005	4.26	0.255/1	66.21	17.52	0.1515 (0.01 N acid)
Butyric	0.01	0.005	4.28	0.282/1	56.58	22.05	0.443 (0.01 N acid)
Valeric	0.009	0.0045	4.26	0.291/1	65.56	26.3	2.60 (0.01 N acid)
Caproic	0.01	0.005	4.27	0.257/1	55.58	30.75	9.47 (0.01 N acid)
Heptylic	0.008	0.004	4.39	0.358/1	40.25	35.7	49.3 (0.01 N acid)
Caprylic	0.003	0.0015	4.88	1.09/1	28.82	39.7	31.9 (0.001 N acid)

[1] Measured against air.
[2] Molecular refraction for Na line D.
[3] Ratio for acid in olive oil to acid in water at equilibrium (Bodansky, '28).

proximate concentration obtaining in the red cell suspension upon dilution in the rapid mixing device. The acid concentration used was determined by experiment as that concentration which upon mixing with the red cell suspension would yield a pH of approximately 4.2. At this pH the erythrocytes were not immediately hemolyzed and the hydrogen ion concentration of the environment had been appreciably increased. Caprylic acid solutions were used at a slightly higher pH because of their lytic action and slight solubility.

It was found experimentally and could be expected from an examination of the oxygen dissociation curve that for a given hydrogen ion concentration a greater amount of oxyhemoglobin dissociated, at oxygen tensions below 60 mm of Hg than at oxygen saturation. In practice beef red cell suspensions and acid solutions were equilibrated to calculated oxygen tensions of 10 mm Hg and human red cells suspensions to 20 mm Hg. The equilibration was accomplished by mounting two rotating tonometers on the laboratory table beside the spectrophotometer. Water displaced the gas mixture by means of a siphon and drove the gas through the tonometers from which it was carried through the reservoirs of the rapid mixing device and finally through a water trap. After equilibration, which was usually accomplished within 25 minutes, the red cell suspension and the acid solution, each of approximately 100 ml, were passed directly into the reservoirs without contact with air. The tonometers were not placed in a constant temperature bath but the room in which the equilibration was done maintained a fairly constant temperature. During any one experiment the temperature was never observed to vary more than 0.5° C. and throughout the period of this study the room temperature ranged from 20° to 23° C.

Following equilibration and transfer of the red cell suspension and acid solution to the reservoirs of the rapid mixing device, a "run" was made. This consisted of 5 or 6 different observations of flow and static transmission values at different reaction times. The mixing distance was maintained constant during a run and the reaction time was varied

by changing the speed of flow of the liquids through the observation tube. In a series of three runs at the same reaction time the difference between flow and static readings, measured as densities (in which the optical density is the reciprocal of the logarithm of the per cent light transmitted), were averaged. These average values were then corrected to a scale of 100 in order to make the different series comparable. Control studies were made using 1% NaCl solutions in place of the acid diluent, in order to determine instrument variation, effects of change of velocity of flow and turbulence. The effects of light scatter were also investigated and considered of negligible importance in this study. When a red cell suspension was made up in 1% NaCl solution and a series of readings on the suspension made, it was found that consecutive readings did not vary more than 0.5%. Concentrations of red cells were used which transmitted approximately 50% of the incident light. Under these conditions, approximately 5% difference in transmission of light occurred between the absence of penetration of an acid and maximum penetration.

It was of importance to this study to know when hemolysis began and something of its course in time. Also because hemolytic techniques had been used as a measure of the penetration of compounds into red cells it was of interest to compare hemolytic data for these acids with the penetration data obtained by the spectrophotometric technique. Therefore, studies with the densimeter apparatus of Parpart ('35) were made for each acid and each type of blood studied using the same acid and blood concentrations as were used in the rapid mixing device.

RESULTS

A summary of typical results obtained is shown in figure 2 for beef blood and figure 3 for human blood. These graphs are semi-logarithmic plots in which the lines were fitted by the method of least squares. Each point on a line is an average of three measurements. The ordinate, corrected density difference, is plotted against time in seconds. The time

referred to is the reaction time previously described. Density differences were obtained by subtracting static density values from flow density values. An average of three such differences for a given reaction time was corrected on the basis of 100 in order to bring the results for different acids on one graph. The failure of the lines for the different acids to have a common origin in both figures 2 and 3, is owing to their having been calculated lines. While the exponential nature of these data may be expected from theoretical considerations,

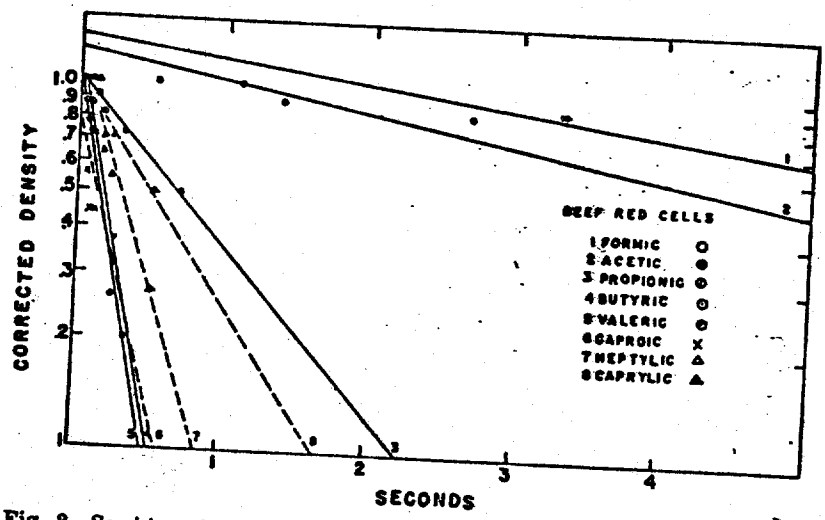

Fig. 2 Semi-logarithmic plot of representative data for beef erythrocytes. The ordinate, corrected density, refers to density differences between flow and static readings in the apparatus.

it was confirmed by plotting the corrected density difference against the reaction time. For convenience of presentation the semi-logarithmic plot only is given here.

Figure 2 shows a progressive increase in the rate of penetration of the acids into beef cells from formic to valeric, with butyric, valeric and caproic acids having much the same slope. The slopes of these three acids are not statistically different. A reverse condition was obtained for the rate of penetration of heptylic acid which was slower than caproic while caprylic

acid is slower than heptylic. Figure 3, for human blood, shows much the same trend as figure 2 for beef blood. The exception to be noted is that caprylic acid has a slightly slower rate of penetration than does heptylic acid. Since the lipid solubility of these acids increases with the length of the carbon chain, the results obtained with heptylic and caprylic acids were sufficiently in disagreement with the Overton theory to suggest a confirmatory study. This was undertaken with a Beckman spectrophotometer which has an altogether different optical and recording system from that of the Coleman 10S.

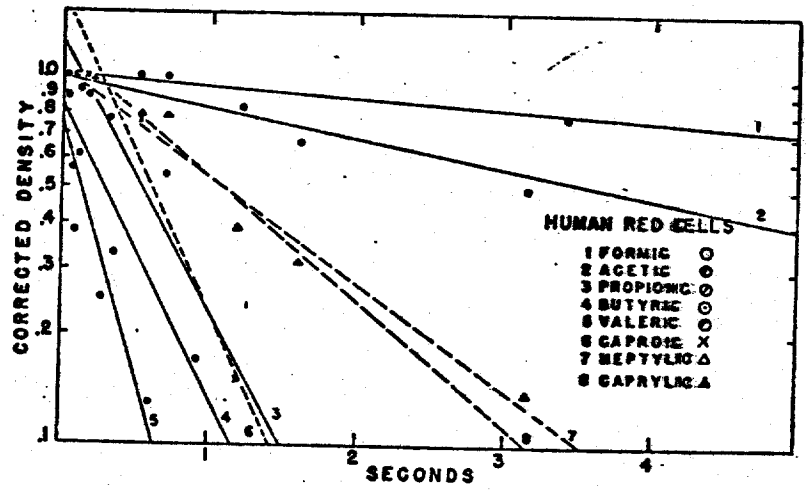

Fig. 3 Semi-logarithmic plot of representative data for human erythrocytes.

The same results were obtained with the Beckman instrument using beef blood only.

The results of the densimeter studies, which were performed for each acid and each type of blood, are plotted in figure 4. These graphs compare time to 50% hemolysis, using the densimeter technique, with time to 50% penetration, using the spectrophotometric technique. The data for the penetration plots were calculated from the relation, $\log C/C_0 = -kt$, where C_0 is the final concentration of acid at equilibrium inside the cells, C, the concentration at any given time, and

$-k$, the slope of the plot of log C/C_0 against t, the time in seconds. When $C = 50$, $C_0 = 100$, and $t = 50$, the following relations exist: $t_{50} = 0.3010/-k$. An examination of figure 4 reveals that the beef and human red cells produce parallel curves for the hemolytic data and parallel curves for the

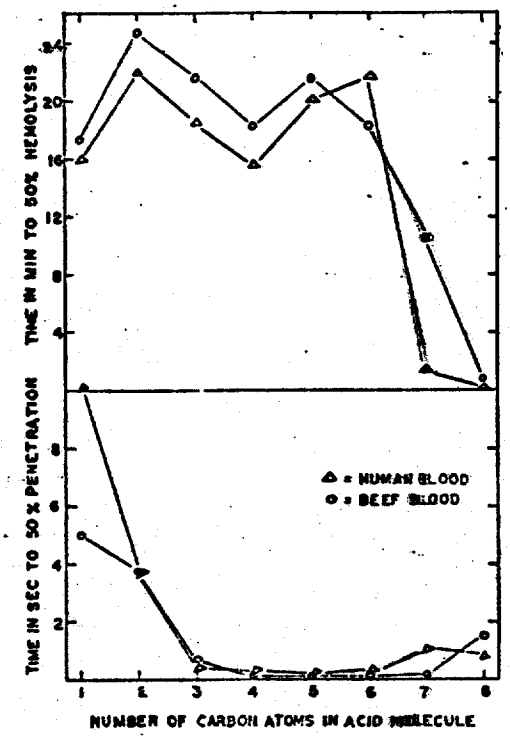

Fig. 4 Comparison between hemolytic and penetration times for each of the acids studied. The hemolysis scale is in minutes and the penetration scale in seconds.

penetration data. However, the two sets of curves are roughly the inverse of each other. In the hemolytic curves, formic acid produces 50% hemolysis about as rapidly as butyric, and one might expect that valeric would be faster than either but this is not the case. In the penetration data the most unexpected result is the decrease in the rate of penetration with

heptylic and caprylic acids when these are known to be the most lipid soluble of the group studied. The times to 50% penetration are listed in table 2 for each acid and each type of blood studied.

TABLE 2

Time to 50% penetration

ACID	BEEF CELLS —k[1]	BEEF TIME IN SEC. TO 50% PENETRATION	HUMAN CELLS —k	HUMAN TIME IN SEC. TO 50% PENETRATION
Formic	0.05969	5.04	0.02964	10.16
Acetic	0.07934	3.79	0.08073	3.73
Propionic	0.4564	0.66	0.7316	0.412
Butyric	2.0816	0.145	0.7949	0.379
Valeric	2.080	0.145	1.3852	0.2175
Caproic	1.5312	0.197	0.8372	0.359
Heptylic	1.2558	0.239	0.2895	1.04
Caprylic	0.6075	0.496	0.3424	0.878

[1] — k = slope of plot from figure 2.

DISCUSSION

A number of investigators have studied the penetration of the lower fatty acids into cells and a few have determined the relative order of penetration of these acids. Harvey ('14a, '14b), taking advantage of a natural indicator in the pigment cells of the holothurian, Stichopus, observed the following order of penetration of the lower fatty acids: valeric = formic > butyric = propionic = acetic. Crozier ('16a, '16b) reported similar results using pigment cells in a mollusc, Chromodoris. He obtained the series: isovaleric > formic > butyric > propionic > acetic. Crozier later ('18) determined the effect of these acids on the sensory activation of the earthworm, Allolobophora, but these results, like those obtained earlier by Loeb ('09, '13) on sea urchin fertilization membrane formation or those of Taylor ('28) on taste threshold of acids in human beings, are difficult to assess because of the absence of evidence for penetration.

Gryns (1896) observed that the ammonium salts of acetic, propionic and butyric acids penetrated horse erythrocytes. Later Jacobs ('26, '27) measured the hemolysis times of ammonium formate, acetate, propionate, butyrate and valerianate in beef erythrocytes. He found an increase in the rate of hemolysis with increasing length of the carbon chain, although some leveling off in the speed of penetration was observed in the butyrate and valerianate salts. He advanced evidence that the hemolysis was produced by the penetration of ammonia and fatty acid molecules following hydrolysis of the salt. Subsequently Jacobs and Parpart ('38) and Jacobs ('40) were able to show that at extreme pH values, either acid or alkaline, the hemolysis was slower than in the neutral range when larger numbers of both species of molecules were available for penetration. Höber ('36) made a study of the hemolysis rate of the sodium and ammonium salts of the lower fatty acids using the photocell method of Höber and Orskov ('33). He found, using red cells of the beef, sheep, rat, man and mouse, that exposure of these cells at pH 7.3 to solutions of the sodium salts of the C_2 to C_6 fatty acids resulted in no detectable alteration of the cells within many hours; however, the C_7 to C_9 fatty acids produced hemolysis within 20 minutes. With the ammonium salts of these acids he obtained the same results as did Jacobs. He further observed the hemolytic rate for the ammonium salts rose among the species studied in the following order: mouse, rat, man < beef < sheep. Dziemian ('39) who repeated this work in part, using the ammonium salts of the C_1 to C_4 fatty acids, was in agreement with it except for some of the species difference observations. He found the rat cells more permeable than the beef cells in every instance studied.

Stewart ('31) investigated the volume changes in sea urchin eggs when exposed to the same series of ammonium salts of fatty acids as Jacobs had studied in beef erythrocytes. She also found an increasing rate of volume change with increasing carbon content of the fatty acid and attributed the differential rate of penetration to the relative ease of solubility

of these acids in the lipids of the egg surface. Bouillenne in 1930 made a study of the penetration of the ammonium salts of the lower fatty acids into epidermal cells of Tradescantia virginica using a deplasmolysis technique. He observed the same order of penetration as had Jacobs and Stewart. In these studies of Jacobs, of Höber, of Stewart and of Bouillenne, the penetration of ammonia molecules must be regarded as a contributing factor in the volume change or hemolysis observed. And while there is no reason to believe that in the absence of ammonia the order of penetration of these acids would be changed, the rate of hemolysis or volume change may be modified. Bodansky ('28, '31, '32, '33) investigated this point in dog and human erythrocytes using solutions of the lower fatty acids in physiological salt solutions. With complete hemolysis as a criterion of penetration rate, he observed an increase in rate of penetration from C_2 to C_{10} fatty acids. He correlated these findings with the relative solubility of these acids in olive oil and associated their penetration into the cells with lipid solubility. Solutions of the acids C_2 to C_6 were used at a pH below 4.0; the solutions of C_7 to C_{10} acids are intensely lytic as shown by Höber ('36). This work of Bodansky's appears to involve what Jacobs ('24) called secondary penetration of acids or penetration with injury. His work does raise the question as to what extent hemolysis may be used as a criterion of penetration of a lytic compound. The work of Parpart and Shull ('35) indicated that in studies with non-lytic compounds such as glycerol, volume changes are closely paralleled by entrance of the glycerol. In the present study a comparison was made between rates of penetration as determined by hemolysis, using the densimeter technique of Parpart ('35) and penetration, as determined spectrophotometrically by the modified Hartridge-Roughton technique. The results are shown in figure 4. The irregularity in the hemolysis curve for the C_4 acid may be owing to evaporation of fluid in the densimeter. However, the hemolysis curves bear no resemblance to the penetration curves, although aside from instrumental differences both curves were

obtained under the same conditions. This graph indicates that rates of hemolysis of red cells by lytic compounds do not parallel rates of penetration of these comopunds as determined by the modified Hartridge-Roughton technique.

The lower graphs of figure 4 show clearly the results of this investigation on beef and human cells. An increase in rate of penetration occurs between C_1 and C_3 acids with C_4, C_5 and C_6 acids exhibiting a nearly constant rate of penetration. The C_7 and C_8 acids penetrate more slowly than the C_4 to C_6 acids. The fact that these studies were made at the pH range 4.2 to 4.8 rather than at neutrality may have influenced the results. However, it was technically impossible to conduct the experiments af pH 7.0 and in any case, the measurements were made in such short periods of time that the lytic action of the higher acids was minimized. In addition to presenting information on the relative rate of penetration of these acids the time to 50% penetration has been calculated and has been found to be quite rapid, varying from 0.14 seconds to over 5.0 seconds as shown by table 2. The technique used in the present study is thought to yield less equivocal data on the penetration of these acids than any of the other methods used in this type of investigation. The fact that the present study has been limited to the blood of two species of mammals and has been made at approximately a single pH value limits the general applicability of the results. However, the results obtained, when their limitations are considered lend only partial support to the published evidence for a continuous series of fatty acids whose rate of penetration into red cells increases with increasing carbon content of the fatty acid.

Some of the physical characteristics of the acids studied in this investigation are listed in table 1. An attempt has been made to correlate the penetration results of this study with some of these characteristics, particularly with concentration, molecular volume or distribution ratio. The C_1 to C_3 acids do appear to penetrate in the order of their relative solubility in olive oil but not in proportion to their solubility. The rates of penetration of the C_6 to C_8 acids do not follow

their relative solubility in olive oil. These data on penetration do not consistently favor the idea that these acids penetrate by reason of their solubility in lipid regions of the erythrocyte and in this respect support the findings of Dziemian ('39). They do suggest that molecular size, in the case of the C_6, C_7 and C_8 acids, may be a limiting factor in the rate of penetration. And it is suggested that the plateau observed in butyric, valeric and caproic acids may represent a balance of two opposite forces, increasing molecular volume, on the one hand, tending to retard penetration and increased solubility in lipid regions, on the other hand, tending to increase the penetration rate.

In considering the significance of the results of this investigation, the fact should be noted that the times for penetration of these acids were measured and were found to be, by comparison with hemolytic times, of a much smaller magnitude. Future work with the method described in this paper should provide information leading to the determination of permeability constants for these acids.

SUMMARY

1. A modification of the Hartridge-Roughton rapid mixing apparatus was developed and used in conjunction with a spectrophotometer to measure the relative rates of penetration of lower fatty acids into beef and human erythocytes. The measurements of penetration depended upon the experimental observation that oxyhemoglobin dissociates to a new equilibrium with an increase in the hydrogen ion concentration within the cell.

2. The relative rates of penetration of the fatty acids investigated were found to be (a) for beef cells: caprylic < heptylic < caproic = valeric = butyric > propionic > acetic > formic; (b) for human cells: caprylic = heptylic < caproic < valeric > butyric > propionic > acetic > formic.

3. The rates of hemolysis by these acids were determined and found to be (a) for beef cells: caprylic > heptylic >

caproic > valeric < butyric > propionic > acetic < formic; (b) for human cells: caprylic > heptylic > caproic < valeric < butyric > propionic > acetic < formic.

4. The times to 50% penetration of these acids were measured and found to be of much smaller magnitude than those determined by hemolytic methods.

5. The results of penetration and hemolysis are discussed in the light of previous hemolytic work done with these acids and the more general studies made of penetration into eggs and tissue cells.

6. An attempt was made to correlate the penetration results with lipid solubilty, surface tension or molecular volume data. The retardation of penetration observed in the C_6, C_7, and C_8 acids suggest that in this homologous series in which lipid solubility increases with increasing carbon content of the acids, molecular volume remains a limiting factor in the penetration rate.

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Metabolism of 1-C^{14} Octanoic and 1-C^{14} Palmitic Acid by Rat Intestinal Slices.* (30565)

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The classic studies of Bloom and Chaikoff (1,2) and Borgström(3) demonstrated that after intestinal absorption, fatty acid molecules with chain lengths of 10 or less carbon atoms were transported primarily in the portal vein whereas fatty acids with chain lengths of greater than 16 carbon atoms were transported predominantly in lymph. Recent studies(4-7) have indicated that differences in the behavior of fatty acids during absorption are probably due to variations in their intramucosal metabolism as well as to differences in physical properties. However, these studies have dealt primarily with the processes of mucosal hydrolysis of glycerides and mucosal esterification of fatty acids. Little attention has been directed at the problem of catabolism of fatty acids by intestinal mucosa.

In the present investigation studies were undertaken to define more clearly the differences in the mucosal metabolism of a long chain fatty acid (palmitic) as compared to a medium chain fatty acid (octanoic). The data obtained indicate that octanoic acid is catabolized to CO_2 and water soluble products to a greater extent than palmitic acid by rat jejunal slices. Analysis of the lipid soluble products by thin layer and gas liquid chromatography revealed additional differences in the metabolism of these 2 fatty acids.

Materials and methods. 1-C^{14} octanoic and 1-C^{14} palmitic acid (New England Nuclear Corp.) were purified by thin layer chromatography. The fatty acid substrates were dissolved in a small amount of ether and homogenized in 10% crystalline bovine albumin. Non-radioactive lipid carrier was added before homogenization so that the final concentration of the lipid-albumin suspensions was 10 micromoles lipid per ml.

Aliquots of the various lipid-albumin suspensions and other water soluble products were added to 12 ml of a counting solution prepared as previously described(8). The solution prepared for counting substances in organic solvents contained 15 ml of toluene with 0.04% liquifier(P... + Ch...

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Radioassay was performed in a Packard Tri-Carb liquid scintillation spectrometer. Quenching was corrected for by utilizing a channels ratio method as described by Bush(9).

Female albino rats (Charles River Breeding Laboratories) weighing 160-200 g were fasted overnight before use in these experiments. The animals were killed by a blow on the head and the small bowel rinsed *in situ* with 50 ml of ice cold 0.85% NaCl. The upper half of the intestine was everted over a glass rod and intestinal slices prepared as previously described(8). Six jejunal slices weighing between .35-.40 g were added to each incubation flask which contained the following: 0.5 ml of 5-fold concentrated low calcium Kreb's-Ringer bicarbonate buffer, pH 7.4, 0.5 ml of 0.15M $NaHCO_3$, 7.5 μmoles of glucose in 1.0 ml water, and 1.0 ml of a lipid-albumin suspension containing either 1-C^{14} octanoic or 1-C^{14} palmitic acid. The flasks were gassed for 30 minutes with 95% O_2, 5% CO_2 prior to addition of the jejunal slices and lipid substrates. In each experiment control flasks were used in which 0.25 ml of 10N H_2SO_4 was added at the start of incubation. The incubations were carried out in stoppered flasks at 37°C for 30 minutes and were terminated either by addition of 0.25 ml of 10N H_2SO_4 or placement in ice.

To measure the conversion of 1-C^{14} octanoic and 1-C^{14} palmitic acid to $C^{14}O$, 0.25 ml of 10N H_2SO_4 was added to stoppered flasks that were cooled in ice for 15 minutes after which 1.0 ml of hyamine 10-X was added to the center wall. The flasks remained in ice for 30 minutes after which the hyamine was removed and aliquots assayed for radioactivity.

When lipid soluble products were analyzed, the reactions were terminated by placing the flasks in ice. The tissue slices were removed, rinsed in distilled water, and blotted dry. The tissue slices and media were extracted separately by the method of Folch *et al*(10). In experiments with palmitic acid as radioactive substrate, the solvents containing the extracted lipids were evaporated to dryness under a nitrogen stream at room temperature and the residues redissolved in chloroform. In roform-methanol added at the end of the incubation contained 42 μmoles of carrier octanoic acid and 6 μmoles of carrier trioctanoin and the pH during the Folch extraction was adjusted to below 3. Because of the volatility of octanoic acid, the solvents containing the lipid extracts were evaporated slowly under a nitrogen stream in an ice bath. The dried extracts were then resuspended in chloroform. Aliquots of the lipid soluble products were assayed for radioactivity and also analyzed by thin layer and gas liquid chromatography.

Gas liquid chromatography analyses were performed using a Barber-Coleman model 25C equipped with an Argon ionization detector. The gas stream was split so that 25% was directed to the mass detector and 75% to the radioactive counter (fraction collector). The inlet pressure was 30 p.s.i. and the flow rate 80 ml per minute. Radioassay was performed by the method of Karmen *et al* (11) utilizing a Packard liquid scintillation spectrometer. For each analysis 5 μg of material in 5 μL was injected into the column. The column was coiled, 6 ft by 1/8 inch in diameter. The liquid phase was either 15% apiezon L or 20% ethylene glycol succinate and the solid support 80-90 mesh anakron. The column temperature was 197°C, the flash heat temperature 285°C, and the cell temperature 200°C.

Results. The data shown in Table I and Fig. 1 indicate that small intestinal slices *in vitro* metabolize 1-C^{14}-octanoic acid different-

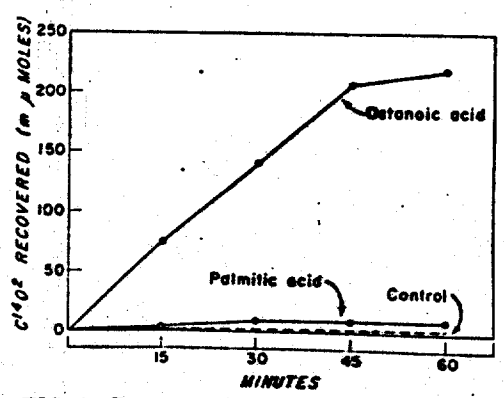

FIG. 1. Recovery of $C^{14}O_2$ after incubation of 1-C^{14} octanoic and 1-C^{14} palmitic acid with jejunal slices. Incubations were carried out for 15, 30, 45 + 60 min under conditions as described in Methods.

TABLE I. Metabolism of 1-C^{14} Octanoic and 1-C^{14} Palmitic Acid to $C^{14}O_2$, Water Soluble, and Lipid Soluble Products by Bowel Slices.

Lipid substrate (10 μmoles)	No. experiments	Distribution of recovered radioactivity (%)			Total recovery of administered radioactivity (%)
		$C^{14}O_2$	Water soluble products	Lipid soluble products	
Octanoic acid	4	1.66 ± .12 *	2.09 ± .8	96.25 ± .8	84–98
Palmitic "	4	.097 ± .003	.106 ± .009	99.80 ± .02	92–98
p value		<.01	<.01	<.01	

The component of incubation mixtures and conditions of incubation were as described in *Methods*.
* Mean ± 1 S.D.

ly than 1-C^{14}-palmitic acid. A significantly greater amount of octanoic acid was oxidized to CO_2 and water soluble products and less was recovered as lipid soluble products compared to palmitic acid. The lipid soluble products from these studies were also analyzed by thin layer and gas liquid chromatography (Tables II and III) and additional differences in the metabolism of octanoic and palmitic acids by jejunal slices demonstrated. The data in Table II indicate that a significantly greater amount of the administered palmitic acid was esterified to triglyceride as compared to octanoic acid (33% *vs* 14%; p = <.01). Furthermore, there was also a greater incorporation of palmitic acid into total neutral lipid compared to octanoic acid (41% *vs* 19%; p = <.01).

The data in Table III indicate that after incubation of C^{14}-palmitic acid with jejunal slices 96% of the label was recovered in the form of palmitic acid as determined by gas liquid chromatography. In contrast, after incubation of C^{14}-octanoic acid with jejunal slices, a small but significant fraction of the label was recovered in fatty acids other than octanoic acid. As indicated in Table III, 3.7% was recovered as decanoic acid and 4.9% was incorporated into fatty acids with chain lengths of 12-20 carbon atoms.

Discussion. A considerable amount of evidence has accumulated indicating that medium chain and long chain fatty acids are metabolized differently by the intestinal mucosa, and this information has recently been summarized(12). It has been demonstrated that a medium chain fatty acid such as octanoic is poorly incorporated into mucosal triglycerides as compared to long chain fatty acids such as myristic, palmitic, and stearic(4-7). This has been shown to be due to several factors: (1) medium chain fatty acids are poorly activated to CoA thiolesters(13,14); (2) medium chain monoglycerides are rapidly hydrolyzed by a mucosal monoester hydrolase(15,16); and (3) medium chain triglycerides may also be hydrolyzed by a mucosal lipase system(7). As a result of the low activity of mucosal esterifying enzymes towards medium chain fatty acids and the presence of an active mucosal hydrolytic system directed towards medium chain monoglycerides and triglycerides, medium chain fatty acids accumulate in the intestinal mucosa predominantly in the form of non-esterified fatty acids regardless of whether medium chain fatty acids *per se* or medium chain triglycerides are administered. In contrast, long chain

TABLE II. Thin Layer Chromatography Analysis of Lipid Soluble Products After Incubation of 1-C^{14} Octanoic and 1-C^{14} Palmitic Acid with Jejunal Slices.

C^{14}-lipid substrate (10 μmoles)	No. experiments	Analysis of	Lipid recovered (μmoles)	Distribution of radioactivity (%)				
				Monoglyceride & phospholipids	Diglyceride	Fatty acid	Triglyceride	Neutral lipid
Octanoic acid	4	Medium	7.836 ± .18*	.2 ± .17	.2 ± .1	99.4 ± .2	.2 ± .1	.6 ± .1
	4	Tissue	.806 ± .09	2.7 ± 1.50	2.6 ± 1.5	80.6 ± 8.5	14.0 ± 5.9	19.4 ± 8.5
Palmitic acid	5	Medium	8.970 ± .2	.4 ± .1	1.2 ± .2	94.6 ± 1.4	3.8 ± 1.3	5.4 ± 1.3
	5	Tissue	.905 ± .08	4.4 ± .3	3.8 ± .5	58.8 ± 7.2	33.0 ± 7.5	41.2 ± 7.5

* Mean ± 1 S.D.

TABLE III. Gas Liquid Chromatography Analysis of Lipid Soluble Products Obtained After Incubation of Jejunal Slices with Either 1-C^{14} Octanoic Acid or 1-C^{14} Palmitic Acid.

Lipid substrate	Analysis of	No. experiments	Distribution of radioactive label in fatty acids (%)										Recovered radioactivity, dpm × 10^{-3}
			8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:0	
1. Octanoic acid	Medium	2	99.0										9.2
	Tissue lipids	3	91.1	3.7	———	———	———	4.9	———	———	———	———	2.8
2. Palmitic acid	Medium	2					99.0		2.0	<1.0	<1.0	<1.0	8.0
	Tissue lipids	4	———	<1.0	———	———	95.6	<1.0					

fatty acids are present in intestinal mucosa primarily in the form of triglycerides after administration of either long chain fatty acids or long chain triglycerides(4-7).

The data obtained in the present study indicate that a significantly greater amount of palmitic acid was esterified to triglyceride and neutral lipids by intestinal mucosa as compared to octanoic acid. However, it is noteworthy that after incubation of 10 μmoles of octanoic acid with jejunal slices, 0.156 μmoles were recovered as neutral lipid (Table II). This value is somewhat higher than has been reported by others(4,5) and could possibly be related to the following factors: (1) tissue slices were used which may be more active than tissue homogenates in the esterification of fatty acid substrate(4); (2) larger amounts of lipid substrate (10 μmoles) were utilized in comparison with previous studies; and (3) cold carrier lipid was used to minimize loss of radioactivity during the extraction process.

It was of interest that octanoic acid was catabolized by intestinal mucosa to a greater extent than palmitic acid. This was also reflected by the observation that 9% of the C^{14} label from octanoic acid was incorporated into fatty acids with chain lengths of 10-20 carbon atoms (Table III). This suggests that some of the octanoic acid may first have been catabolized to 2 carbon fragments (*i.e.*, acetate) which were subsequently incorporated into long chain fatty acids. Since medium chain fatty acids are poorly activated to CoA thiolesters(13,14), it seems unlikely that the octanoic acid underwent elongation reactions to yield the longer chain fatty acids. Invest., 1964, V 43, 878.

Summary. Measurements were carried out on the *in vitro* metabolism of C^{14}-octanoic and C^{14}-palmitic acid by rat intestinal slices. Significantly more octanoic acid was catabolized to CO_2 and water soluble products and less recovered as lipid soluble products compared to when palmitic acid was the substrate. Analysis of these lipid soluble products by thin layer chromatography revealed that more of the palmitic acid was esterified to triglyceride compared to octanoic acid. Further analysis of the lipid soluble products by gas liquid chromatography demonstrated that a small but significant amount (9%) of the label from C^{14}-octanoic acid was recovered in fatty acids with chain lengths of 10-20 carbon atoms. This suggests that some of the C^{14}-octanoic acid may have been catabolized to C^{14}-acetate which was then incorporated into long chain fatty acids. In contrast, virtually all of the label from C^{14}-palmitic acid was recovered as such, indicating that very little mucosal catabolism of palmitic acid had occurred.

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The Presence of Lymphocytes in Long Term Cultures of Newborn Mouse Thymic Epithelium.* (30566)

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Observations of *in vitro* cultures of newborn thymus fragments followed for as long as 120 days have been reported(1). We will report here the observations on cultures derived from newborn mouse thymus fragments initially explanted in a plasma clot and followed for over 22 months. The term epithelial cell, instead of that of reticulum cell employed in our previous work, will be used in the present note to describe the cells originating from the epithelial reticulum of the thymus. This appears justified both by the morphological appearance of the cells observed in culture and by the embryological derivation of the thymus(2,3,4,5,6,7).

Material and methods. Source of the thymus was a 24-hour-old MA mouse. The mouse was killed with ether and the thymus aseptically removed to a Petri dish containing Hanks' BSS. After washing, the thymus was transferred to a second Petri dish containing Hanks' BSS and finely minced. The minute fragments were then transferred to 2 Leighton tubes (Belco Glass Co., Vineland, N. J.) into which a coverslip had been previously inserted. Two drops of chicken plasma (Difco) were pipetted to the coverslip before inserting the thymus fragments into the tube. The plasma solidified rapidly after transfer of the fragments and the medium was then added. This procedure favored the adhesion of the fragments to the glass.

The culture medium was Hanks' BSS with phenol red as indicator, plus vitamins, glutamin, essential amino acids and was supplemented with 11% fetal bovine serum (Microbiological Associates, Bethesda, Md.). No antibiotics were used. The medium was first changed after 4 days and thereafter changed every 5-7 days. Every 2 changes, after the old medium was discarded, the culture was washed with Hanks' BSS before the fresh medium was added.

Of the original cultures, one was sacrificed

FIG. 1. 4-day-old culture. Macrophages phagocytizing lymphocytes and debris, surrounded by dead or degenerating lymphocytes. 250 ×.
FIG. 2. 14-day-old culture, large lymphocytes growing on background of a continuous sheet of epithelial cells. Original magnification 250 ×, enlarged 3 times. Phase contrast.
FIG. 3. 14-day-old culture. Lymphocytes growing on epithelial cells. May-Grünwald and Giemsa, 250 ×.
FIG. 4. 15-month-old culture. Mitosis of an epithelial cell. Original magnification 250 ×, enlarged 3 times. Phase contrast.
FIG. 5. 22-hour-old subculture from the 14-month-old original culture. Colony of epithelial cells. May-Grünwald and Giemsa, 110 ×.
FIG. 6. 18-hour-old subculture from the 14-month-old original culture. Small colony of epithelial cells. A few lymphocytes with pyknotic nuclei are visible. One is engulfed in a cytoplasmatic vacuole. May-Grünwald and Giemsa, 250 ×.

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FATTY ACID METABOLISM OF RAT DIAPHRAGM*

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Recent studies (1, 2) have indicated that hepatectomized animals have the ability to oxidize fatty acids directly to carbon dioxide. We have obtained evidence which indicates that rat diaphragm oxidizes exogenous octanoic acid *in vitro*. Inasmuch as the feeding of diets high in fat has been found to limit subsequent utilization of glucose by the diaphragm (3), the effect of diet on this oxidation of octanoate has been studied.

EXPERIMENTAL

All the animals used were of a Sprague-Dawley strain. Stock animals were maintained on a standard Purina fox chow diet. In the cases in which the caloric intake was principally fat or carbohydrate, however, the diets were identical with those used in a previous study (3). The animals were sacrificed by decapitation, and their diaphragms removed and incubated under an atmosphere of oxygen in Krebs-Ringer phosphate buffer at pH 7.4 with other additions as indicated. The octanoic acid used was redistilled from a sample obtained from the Eastman Kodak Company. The neutral equivalent of the acid and the melting point of its amide derivative agreed with the theoretical values. Octanoic acid was determined by the method of Lehninger and Smith (4).

Octanoate Uptake by Diaphragm from Animals Fed High Carbohydrate and High Fat Diets—The hemidiaphragms from animals in each group were paired between a control flask and a flask containing octanoate in the side arm. After equilibration, octanoate was added to the main compartment, and incubation continued for 2 hours. The final octanoate concentration in the medium was determined and used to calculate the octanoate uptake per 100 mg. of fresh tissue. In all the experiments, at the 2.0 and 3.3 μM level of substrate (Table I), the carbohydrate tissues removed more octanoate from the medium than did the fat tissues. Octanoic acid significantly increased oxygen consumption of all tissues during the period observed.

* This investigation was supported in part by a research grant from the Williams-Waterman Fund for the Combat of Dietary Diseases of the Research Corporation, New York. It is a pleasure for the authors to acknowledge indebtedness to Dr. Leo T. Samuels of the Department of Biochemistry of the University of Utah.

Octanoate Oxidation by Diaphragm from Animals Fed Stock Diet—Stock animals weighing from 150 to 250 gm. were sacrificed. The diaphragm was removed; one hemidiaphragm was taken as a control, and the other

TABLE I

Uptake of Octanoic Acid and Oxygen by Diaphragm from Rats Fed High Fat and High Carbohydrate Diets

Each experiment includes hemidiaphragms from three animals for each dietary group. The tissues from each rat were paired between a control flask and a flask containing octanoate in the side arm. After equilibration and addition of substrate, the tissues were incubated 120 minutes as usual. The oxygen consumption is reported for the first 60 minute period only. The results are expressed as micromoles per 100 mg. of wet tissue.

Experiment No.	Octanoate concentration	Oxygen uptake				Octanoate uptake	
		Carbohydrate diet		Fat diet		Carbohydrate diet	Fat diet
		Control	Octanoate	Control	Octanoate		
	μM per ml.						
1	2					0.9	0.8
2	2					1.2	0.6
3	2	6.3	7.0	5.8	6.3	1.0	0.8
4	2	6.2	7.0	5.8	6.4	1.0	0.8
5	2	7.0	7.9	6.1	7.7	1.4	1.0
6	3.3					1.3	0.8
7	3.3	6.8	8.4	6.6	7.3	1.4	1.1
8	3.3	6.3	6.5	5.8	6.7	0.9	0.8
9	3.3	7.0	8.8	6.1	7.7	1.5	1.3
10	3.3	5.5	6.7	5.8	6.0	1.4	1.0
11	6.3	5.6	6.8	4.9	5.8	1.4	1.3
12	6.3	6.8	7.3	6.6	7.4	1.9	1.2
13	6.3	5.5	6.5	5.8	7.1	1.5	2.0
Average ± s.e.m.*.....		6.3 ±0.19	7.3 ±0.25	5.9 ±0.15	6.8 ±0.07	1.3 ±0.08	1.0 ±0.10
Student's *t*.....		5.93†		5.93†		3.06†	

* Standard error of mean.
† Highly significant difference.

incubated in the presence of octanoate. The octanoic acid was added from the side arm to give a final concentration from 0.5 to 5.0 μM per ml. of medium. Oxygen consumption is expressed as micromoles of oxygen removed from the medium per 100 mg. of tissue for the first and the second parts of the 120 minute incubating period. The presence of substrate increased the oxygen consumption in all experiments except one for

the first 60 minute period (Table
ate, this stimulation continued t
levels of octanoate usually inhibit
phase of the incubation.

T

Effect of Octanoic Acid on

The tissues from stock rats were p
phate buffer and a flask containing b
pressed as micromoles per 100 mg. of

Octanoate concentration	First 6 Oxygen Control
0.5	5.0
0.5	6.6
1.0	6.5
1.0	7.4
3.3	5.2
3.3	5.1
3.3	5.2
3.3	5.2
3.3	6.0
3.3	5.8
3.3	6.2
3.3	5.6
3.3	5.9
4.0	6.0
4.0	6.1
4.0	6.3
5.0	5.2
Average ± s.e.m.....	5.8 ±0.16
Student's *t*.....	6.12*

* Highly significant difference.

Time Course of Octanoate Uptake
from six stock rats were removed as
into three approximately equal porti
distributed in six flasks. One flask
were equilibrated. Sodium octanoa
give a concentration of 4.0 μM per
indicated, the flasks were removed,

from Animals Fed Stock Diet—Stock p. were sacrificed. The diaphragm was taken as a control, and the other

I

hragm from Rats Fed High Fat and High ate Diets

agms from three animals for each dietary red between a control flask and a flask equilibration and addition of substrate, as usual. The oxygen consumption is re- . The results are expressed as micromoles

Oxygen uptake			Octanoate uptake	
	Fat diet		Carbohydrate diet	Fat diet
ate	Control	Octanoate		
			0.9	0.8
			1.2	0.6
7.6	5.8	6.3	1.0	0.8
7.0	5.8	6.4	1.0	0.8
	6.1	7.7	1.4	1.0
			1.3	0.8
8.4	6.6	7.3	1.4	1.1
6.5	5.8	6.7	0.9	0.8
	6.1	7.7	1.5	1.3
	5.8	6.0	1.4	1.0
6.8	4.9	5.8	1.4	1.3
7.3	6.6	7.4	1.9	1.2
	5.8	7.1	1.5	2.0
7.3	5.9	6.8	1.3	1.0
0.05	±0.15	±0.07	±0.08	±0.10
	5.93†		3.06†	

ate. The octanoic acid was added ntration from 0.5 to 5.0 μM per ml. xpressed as micromoles of oxygen mg. ssue for the first and the sec- ating period. The presence of sub- t in all experiments except one for

the first 60 minute period (Table II). At 0.5 and 1.0 μM levels of octanoate, this stimulation continued throughout the incubation period. Higher levels of octanoate usually inhibited oxygen consumption during the latter phase of the incubation.

TABLE II

Effect of Octanoic Acid on Oxygen Uptake of Rat Diaphragm

The tissues from stock rats were paired between a control flask containing phosphate buffer and a flask containing buffer plus octanoic acid. The results are expressed as micromoles per 100 mg. of wet tissue.

Octanoate concentration	First 60 min.		Second 60 min.		
	Oxygen uptake		Oxygen uptake		Per cent changes
	Control	Octanoate	Control	Octanoate	
0.5	5.0	6.5	4.4	5.3	+20
0.5	6.6	7.5	6.2	6.9	+11
1.0	6.5	8.6	6.2	7.7	+24
1.0	7.4	9.0	6.7	8.5	+27
3.3	5.2	6.0	4.8	5.4	+13
3.3	5.1	7.3	4.9	5.6	+14
3.3	5.2	5.5	4.9	4.4	−10
3.3	5.2	6.2	4.9	3.2	−32
3.3	6.0	6.7	6.1	4.2	−31
3.3	5.8	7.2	5.2	4.8	−8
3.3	6.2	5.9	5.7	4.3	−25
3.3	5.6	7.2	5.3	4.4	−17
3.3	5.9	6.4	5.6	5.4	−4
4.0	6.0	6.3	6.6	5.3	−20
4.0	6.1	6.7	6.4	5.5	−14
4.0	6.3	6.9	6.6	5.7	−14
5.0	5.2	6.3	4.2	2.7	−36
Average ± s.e.m.	5.8 ±0.16	6.8 ±0.22			
Student's *t*	6.12*				

* Highly significant difference.

Time Course of Octanoate Uptake by Rat Diaphragm—The diaphragms from six stock rats were removed as usual; each hemidiaphragm was cut into three approximately equal portions, and the resulting segments were distributed in six flasks. One flask was boiled for 2 minutes, then all were equilibrated. Sodium octanoate was added from the side arm to give a concentration of 4.0 μM per ml. of medium, and, at the intervals indicated, the flasks were removed, and the remaining octanoic acid

was determined. There was an initial period of rapid removal of octanoate from the medium (Fig. 1). This rate decreased markedly with time. Although no oxygen was consumed by the boiled tissue, it appears that

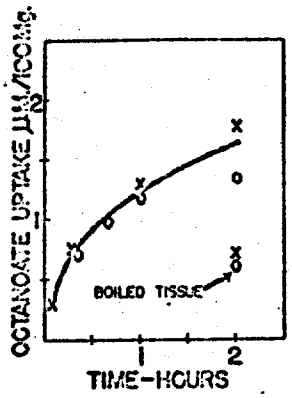

FIG. 1. Stock rats were used in this study. The hemidiaphragms from six animals were cut into three equal portions each and the resulting segments were distributed in six flasks. Octanoic acid was added from the side arm to give a concentration of 4.0 µM per ml. of medium. At the intervals indicated, the flasks were removed and the remaining octanoate was determined. The results of two similar experiments are given.

TABLE III

Effect of Acetate on Oxygen Consumption and Glycogen Formation of Rat Diaphragm

The tissues from stock rats were paired between a control flask containing glucose in phosphate buffer at a level of 0.05 per cent and a flask containing the same concentration of glucose plus 0.1 per cent acetate (17.2 µM per ml.) in a phosphate buffer. The results are expressed as micromoles per 100 mg. of wet tissue.

Experiment No.	Oxygen consumption			
	First 60 min. period		Second 60 min. period	
	Control	Acetate	Control	Acetate
1	7.0	8.6	5.7	7.7
2	7.0	7.7	6.0	6.7
3	7.3	7.9	6.0	7.1
4	6.4	7.3	5.4	6.9
5	6.3	7.6	5.4	7.6
6	6.3	7.0	5.4	6.9
7	6.7	8.4	5.7	8.0
Average ± s.e.m.	6.7 ±0.15	7.8 ±0.16	5.7 ±0.1	7.3 ±0.19
Student's *t*	6.22*		7.24*	

* Highly significant difference.

approximately 0.7 µM of octanoate
fore, appears to have an appreciabl
Effect of Acetate on Rat Diaphra
gen consumption of diaphragm by
study, and an inhibition of oxygen
Parnes and Wertheimer (5), an att
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ulation is quite similar to that
octanoate.

DISC

Data, presented by Parnes and
indicated that the oxygen consumpt
acetate. These data are in direct
tory. It should be noted that the
sumption reported here give inferen
acetate. A more detailed study of
glycogen change is in progress.

The decreased octanoate uptake
the fat diet is difficult to understand
effect. Octanoate is bound by boi
more vigorously by surviving diaph
binding, the presence of comparat
limit further binding. Of course, t
zymatic difference.

The inhibition of oxygen consu
octanoate in the latter phases of t
well known detergent properties of t
becomes apparent about midway in
uptake curve demonstrates that the
dium is approaching a maximum at

SUM

1. Octanoic acid in relatively lo
consumption by rat diaphragm. Wh
3 µM per ml. of medium, however, an
stimulation.

2. Acetate at a level of 17.2 µM per
consumption of the isolated rat diap

3. The isolated diaphragms from

approximately 0.7 μM of octanoate was bound. The tissue itself, therefore, appears to have an appreciable affinity for octanoate.

Effect of Acetate on Rat Diaphragm—In view of the stimulation in oxygen consumption of diaphragm by octanoate, which was observed in this study, and an inhibition of oxygen consumption by acetate, reported by Parnes and Wertheimer (5), an attempt was made to duplicate their experiment. Glucose and acetate were added to the medium at the levels indicated by Parnes and Wertheimer, and oxygen consumption was measured. In all the experiments (Table III), acetate increased the oxygen consumption of diaphragm throughout the incubation period. This stimulation is quite similar to that observed with low concentrations of octanoate.

DISCUSSION

Data, presented by Parnes and Wertheimer (5) without comment, indicated that the oxygen consumption of rat diaphragm was inhibited by acetate. These data are in direct contrast to the findings in this laboratory. It should be noted that the effects of octanoate on oxygen consumption reported here give inferential support to our experiments with acetate. A more detailed study of the effect of acetate and octanoate on glycogen change is in progress.

The decreased octanoate uptake by the tissues from the animals fed the fat diet is difficult to understand except in terms of a physicochemical effect. Octanoate is bound by boiled tissue and could be bound even more vigorously by surviving diaphragm. If protein plays a part in this binding, the presence of comparatively large amounts of lipide could limit further binding. Of course, these results could also reflect an enzymatic difference.

The inhibition of oxygen consumption with high concentrations of octanoate in the latter phases of the incubation may be related to the well known detergent properties of this type of molecule. The inhibition becomes apparent about midway in the incubation period. This time-uptake curve demonstrates that the removal of octanoate from the medium is approaching a maximum at that time.

SUMMARY

1. Octanoic acid in relatively low concentrations stimulates oxygen consumption by rat diaphragm. When the original concentration exceeds 3 μM per ml. of medium, however, an inhibition usually follows the initial stimulation.

2. Acetate at a level of 17.2 μM per ml. of medium stimulates the oxygen consumption of the isolated rat diaphragm.

3. The isolated diaphragms from animals, previously maintained on a

high carbohydrate intake, remove more octanoate from the medium th do similar tissues from animals fed a fat diet. Even so, tissues fro both groups of animals consumed more oxygen when octanoate w present.

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A NEPHELOMETRIC DETERMINATION

By R. G. HANSEN, W. J. RUT

(*From the Laboratory of Chemistry, Depar Illinois, Urban*

(Received for publication,

The most widely accepted procedure pears to be that of Good, Kramer, an inciples used by Bernard (2). The t ueous potassium hydroxide. Advant lity of glycogen in aqueous alcohol to s the digest and to bring about further ns. The sample is finally hydrolyzed ated from the reducing power of the s This paper reports a new procedure for incipal advantage of which is that a dec required. The turbidity of suspensi hol is the basis of the method suggested i

Solutions—

Potassium hydroxide; 30 gm. of (ved in 100 ml. of distilled water.

Sodium chloride; 2 per cent aqueous.

Ethanol; 95 per cent.

Dreft; 1 per cent aqueous.

Precipitation solution; 1 part of 2 per cent ethanol.

Alcohol reagent; 210 ml. of 95 per cent Cl, 30 ml. of 1 per cent Dreft, and 30 ml.

Procedure

ssue containing from 0 to 2 mg. of glycog ing in a centrifuge tube in 30 per cent tes in a boiling water bath. For rat di of tissue are placed in 0.5 ml. of base. the mixture of 1 part of 2 per cent sodi er cent ethanol. The contents of the tu of air through a capillary. The tube ath until bubbles are observed in the diges tor for at least 3 hours to allow complete

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PORTAL ABSORPTION OF FATTY ACIDS IN LYMPH- AND PORTAL VEIN-CANNULATED RATS

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(Received July 21st, 1966)

SUMMARY

The route and the rate of the intestinal absorption of [1-^{14}C]oleic, [1-^{14}C]-caprylic, and 2-[^{14}C]ethyl-*n*-caproic acids have been studied using lymph- and portal vein-cannulated rats. It was found that 85% of absorbed oleic acid was transported *via* lymphatic system. However, as much as 15% of absorbed oleic acid was transported directly *via* the portal system. With short-chain fatty acids, between 94–98% of the absorbed acids were transported *via* the portal system. Studies on the rate of the intestinal absorption of these acids indicated that 2-ethyl-*n*-caproic acid was absorbed less completely and subsequently metabolized less effectively than the corresponding straight-chain fatty acid, caprylic acid.

Studies on the distribution of radioactivity in lymph lipids showed that most of the radioactivity (85%) was present as triglycerides when [^{14}C]oleic acid was administered to lymph and portal vein fistula rats. However, when the ^{14}C-labeled short-chain fatty acids were given, 96–102% of the radioactivity was present as free fatty acids.

Studies on the distribution of radioactivity in lipid fractions of portal vein blood showed that 50 and 98–100% of the radioactivity present were in the form of free fatty acids when [^{14}C]oleic acid and ^{14}C-labeled short-chain fatty acids, respectively, were administered to lymph and portal vein fistula rats.

INTRODUCTION

It has been reported that long-chain fatty acids, when administered to rats, are absorbed *via* the lymphatic system mainly in the form of triglycerides in chylomicrons[1–8], while short-chain fatty acids are absorbed *via* the portal system and appear there primarily as the free fatty acids[9–11]. However, after administration of radioactive long-chain fatty acids to lymph-fistula rats, small amounts of $^{14}CO_2$ were found in expired air, suggesting possible portal absorption of these fatty acids[1,6*]. According to BORGSTRÖM[12], only about 25% of the absorbed ^{14}C activity appeared

* R. H. COOTS, personal communication.

in the intestinal lymph after the administration of [1-^{14}C]palmitic acid to bile-fistula rats. He suggested that bile salts may effect fat absorption not only by their detergent action in the intestinal lumen, but by directly affecting the metabolism of the mucosal cells, and thereby altering the route and form of fatty acid transport. Recently, SAUNDERS AND DAWSON[13] reported that about one-half of the fed ^{14}C activity was absorbed when uniformly ^{14}C-labeled oleic acid was administered to bile-fistula rats. Moreover, only about 7% of the absorbed ^{14}C activity was recovered in the lymph lipids, and the amount of unesterified fatty acid present in the lymph was 3 to 4 times that in normal animals. These results suggested that in the absence of bile, there is an altered route of transport of absorbed oleic acid as well as some failure in the esterification of the fatty acid. These authors pointed out that, although long-chain fatty acids are primarily transported *via* the lymphatic system after esterification in the mucosa, it was likely that some long-chain fatty acid is normally absorbed *via* the portal vein.

Thus, indirect evidence, such as incomplete recovery of absorbed long-chain fatty acids in lymph[4-8], the presence of $^{14}CO_2$ in expired air of lymph-drained animals fed isotopic long-chain acids[1,6*], and the appearance of radioactivity in portal vein blood of bile-fistula animals[14] have raised some questions regarding the exclusive lymphatic transport of long-chain fatty acids.

In the present experiments, the route and rate of the intestinal absorption of [1-^{14}C]oleic acid, [1-^{14}C]caprylic acid and 2-[^{14}C]ethyl-*n*-caproic acid were studied in lymph- and portal vein-fistula rats over an 8-h period. The lipid distribution in lymph and portal vein blood of the absorbed fatty acids was also determined to provide additional information with respect to the significance of portal absorption of long-chain fatty acids.

MATERIALS AND METHODS

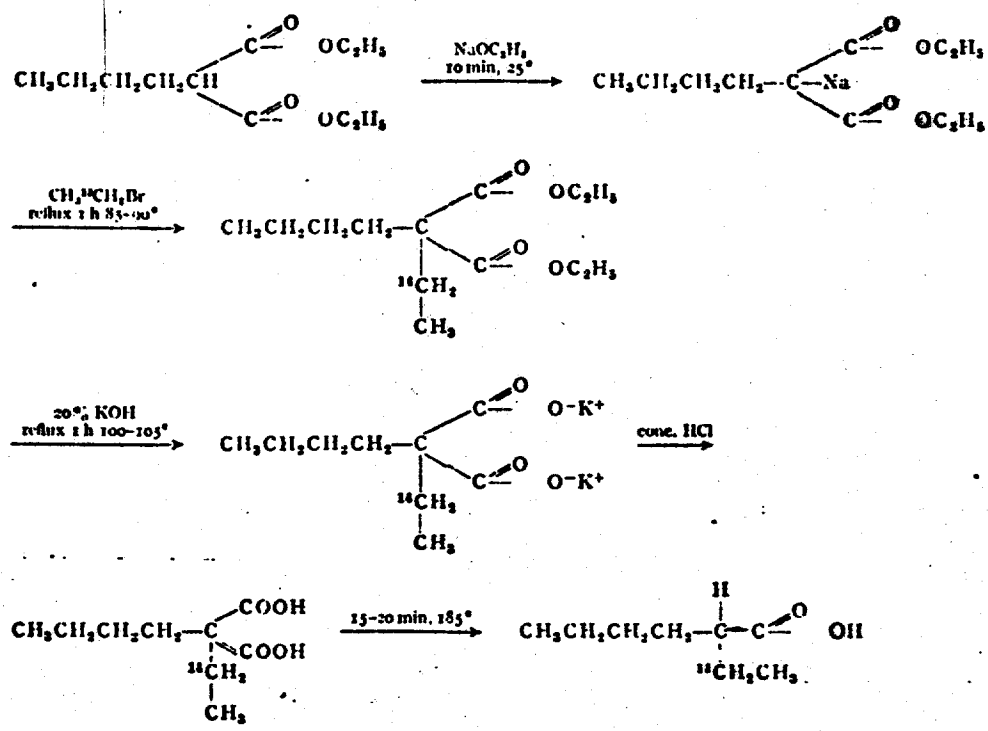
Materials

[1-^{14}C]Oleic acid and sodium [1-^{14}C]caprylate were purchased from the Volk Radio Chemical Company and were diluted with analytically pure (99%) oleic and caprylic acids purchased from Hormel Institute, University of Minnesota. 2-[^{14}C]-ethyl-*n*-caproic acid was synthesized using [1-^{14}C]ethyl bromide and diethylbutyl malonate by the scheme on page 298.

The product contained 10–15% caproic acid and was further purified by distillation and silicic acid chromatography. The final product was obtained 98–99% pure, based on thin-layer chromatography and on refractive index, in comparison with authentic 2-ethyl-*n*-caproic acid. The radiopurity of the three acids was further checked by addition to cold samples of lymph and portal blood and subsequent analysis by the detailed procedures outlined below.

Animals

Adult male rats of Carworth Farm's Wistar strain, weighing 250 to 300 g were maintained on standard laboratory chow and water *ad libitum* prior to operation. The thoracic duct was cannulated by a modification of the procedure of BOLLMAN, CAIN AND GRINDLAY[15]. After completion of the thoracic duct cannulation, the liver and left kidney were replaced in their original positions. The spleen was then freed from the stomach by removing the mesentery between these two organs. The lienal



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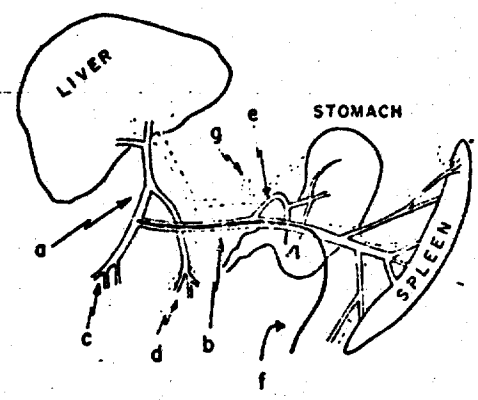

a = portal vein
b = lienal (splenic) vein
c = inferior pancreatico-duodenal vein
d = superior pancreatico-duodenal vein
e = left gastric vein
f = polyethylene tubing
g = celiac artery (from aorta)

Fig. 1. Diagram showing the position of the portal vein fistula.

(splenic) vessels (vein as well as artery) were ligated about one-quarter of an inch from the point of junction of the lienal vein with the left gastric vein, and a second loose ligature was placed below the first. Polyethylene tubing (PE 10), about 8 inches long, was inserted into the lienal vein so that the tip of the tubing extended into the

portal vein but
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Methods

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portal vein but did not block portal blood flow. The ligature was then tightened. Care was taken during the entire procedure not to damage the pancreatic tissue in the gastrosplenic ligament. A small amount of heparin (0.01–0.2 ml, 1000 units per ml) was placed in the tubing to prevent blood clotting. For collection of samples which were used in section 2, distilled water was substituted for heparin. The external end of the tubing was then blocked with a needle. The needle was removed from the tubing only when blood samples were taken. Finally, all blood vessels connected to the spleen were tied off individually to avoid any possible damage to the pancreas, and the spleen was removed. The viscera were then replaced. During the entire period of the operation, the organs were kept moist with warm physiological saline. The final position of the cannulae and tissues are shown in Fig. 1. The animal was placed in a restraining cage designed for collection of feces as well as lymph and blood samples. All animals were allowed free access to saline, but no food for 45 to 50 h prior to the administration of the test emulsions. The test emulsions were prepared immediately before use, as described by VAHOUNY, FAWAL AND TREADWELL[16]. Each of the rats in all groups was given 3 ml of the appropriate test emulsion intragastrically using light vinyl ether anesthesia. After administration of the test emulsion, lymph was collected in 2-h periods for 8 h in tubes containing 0.05 ml of heparin, and 0.1 ml of portal vein blood was collected at 0.25, 0.5, 1, 2, 4, 6 and 8 h. At the end of the experiment, the animals were sacrificed, the stomach and intestine were removed, flushed with saline, and the washings combined with the fecal samples. The combined feces and intestinal contents, and the small intestine were homogenized in 15 ml isotonic saline.

Methods

Aliquots of lymph and blood samples and of the homogenates were extracted with 24 volumes of ethanol–ether (3:1, v/v). 1–2 ml of the ethanol–ether extract was then transferred into a counting vial, 10 ml of liquid scintillant (100 mg 1,4-di-2(5-phenyl oxazolyl)-benzene + 4 g 2,5-diphenyl oxazole per 1000 ml toluene) was added and the radioactivity measured in a liquid scintillation counter (Nuclear Chicago Series 720, 3-channel counter).

For the determination of the form of radioactivity present in the lymph lipids, the lymph samples (4 time samples were taken from each animal) were pooled and total lymph lipids were extracted with 24 volumes of ethanol–ether (3:1, v/v). An aliquot of the ethanol–ether extract was then placed to a large separatory funnel, equal volumes of solution A (distilled water saturated with petroleum ether and diethyl ether and containing 1% $NaHCO_3$ and 0.58% NaCl) and solution B (petroleum ether saturated with distilled water) were added, the contents mixed and allowed to separate. The petroleum ether upper layer was separated from the lower layer and the aqueous layer was returned to the separatory funnel. The same amount of solution B was added, mixed and the upper layer was again separated from the lower layer. The petroleum ether layers contained all of the lymph lipids except free fatty acids and the lower aq. layer contained sodium salts of fatty acids. The petroleum ether layers were then pooled, evaporated to dryness, and the residue dissolved in 30–40 ml of warm petroleum ether. The petroleum ether solution was dried over Na_2SO_4 overnight and the lymph lipids were fractionated by silicic acid column chromatography using a modification of the procedure of HIRSCH AND AHRENS[17].

Aliquots of the total lipid, petroleum ether, and aqueous extracts, and each lipid fraction were taken for the determination of radioactivity.

For the determination of the form of radioactivity present in portal vein blood after the administration of test emulsions containing ^{14}C-labeled fatty acids the samples were treated as follows: 1 h after the administration of the test emulsion, 2 to 3 ml of blood was taken from portal vein using an oxalated syringe. The blood samples were then processed as described above except the lipids were fractionated by thin-layer silicic acid chromatography[18] using a solvent system of hexane–acetone–acetic acid (90:11:3, v/v/v). In all experiments, apparent differences were analyzed for significance using the *t*-test[19].

RESULTS AND DISCUSSION

Before studying the portal absorption of fatty acids, it was necessary to demonstrate the absence of lympho-portal and lympho-vena cava anastomoses in the experimental animals. Two lymph- and portal vein-fistula rats were each given 15 μC [1-^{14}C]oleic acid (292 mg) and 50 μC [7-^{3}H]cholesterol (50 mg), which is known to be absorbed exclusively *via* the lymphatic system. 3 h after the administration of the test emulsion, 2–3 ml of portal vein blood was removed and the radioactivity present was determined. If 0.001% of the administered cholesterol had been transported to the circulatory system by any of the above channels, it could have been easily detected in the portal blood samples. Actually the data showed that whereas a considerable amount of ^{14}C activity was present in the portal vein blood, no ^{3}H activity above background could be detected. Also, in order to obtain additional evidence for portal absorption under normal conditions, a test emulsion containing [1-^{14}C]oleic acid was administered to two lymph and portal vein fistula rats. 1 h later simultaneous samples of portal and inferior vena cava blood were taken and the ratio of lipid radioactivity in the portal blood to inferior vena cava blood was determined. The ratios obtained were 1.5 and 3.2 in the two animals, again indicating absorption and transport *via* the portal system. From these results, it was concluded that ^{14}C activity recovered from portal vein blood after the administration of ^{14}C-labeled fatty acids to the experimental animals was due to direct absorption *via* the portal system.

The rate and route of the intestinal absorption of fatty acids

The rate and route of the intestinal absorption of fatty acids were studied by administering ^{14}C-labeled fatty acids and following the serial appearance of radioactivity in thoracic duct lymph and the portal vein blood. The compositions of the test emulsions used are shown in Table I. The three groups of rats shown in Tables II–III and Figs. 1, 2 are numbered corresponding to the emulsions administered.

As shown in Table II, when [1-^{14}C]oleic acid was administered to lymph and portal vein fistula rats, about 62% of the fed radioactivity was recovered in thoracic duct lymph during the 8-h experimental period, while 23% was unabsorbed and was recovered in feces and intestinal contents. In the groups fed ^{14}C-labeled short-chain acids (Groups 2 and 3), less than 4% of the fed radioactivity was recovered in thoracic duct lymph in 8 h. With [1-^{14}C]caprylic acid 5% of the isotope was recovered in the combined feces and intestinal contents, and less than 1% remained in the intestinal wall at 8 h. However, in the group fed 2-[^{14}C]ethyl-*n*-caproic acid, 48% of the fed

TABLE I

COMPOSITION OF THE INTRAGASTRIC TEST EMULSION

Emulsion number	*1*	*2*	*3*
Albumin, 50 mg	+	+	+
Sodium taurocholate, 288 mg	+	+	+
[1-^{14}C]Oleic acid, 292 mg (4.6 μC)	+		
[1-^{14}C]Caprylic acid, 150 mg (3.0 μC)		+	
2-[^{14}C]Ethyl-*n*-caproic acid, 150 mg* (3.2 μC)			+
0.9% Saline to 3 ml	+	+	+

* ^{14}C label was on the side-chain as shown: $CH_3CH_2CH_2CH_2CHCOOH$ with $^{14}CH_2CH_3$ on the C-2 carbon.

TABLE II

RECOVERY OF RADIOACTIVITY AFTER THE ADMINISTRATION OF ^{14}C-LABELED FATTY ACIDS

Apparent portal absorption was calculated by subtracting the percentage of ^{14}C activity present in thoracic duct lymph, small intestine, and feces and intestinal contents from 100.0%.

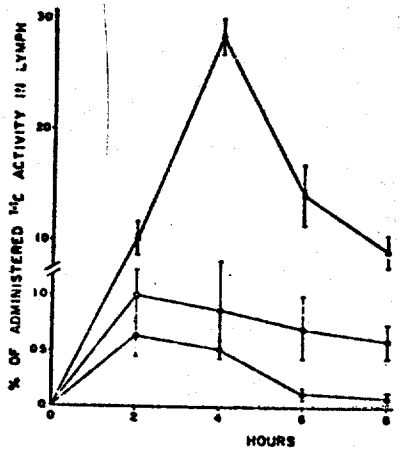
Lymph (8 h) (%)	*Small intestine (%)*	*Feces and intestinal contents (%)*	*Apparent portal absorption (%)*
*Group 1: 292 mg [^{14}C]oleic acid**			
61.9 ± 6.9	3.6 ± 0.8	23.1 ± 2.0	11.3 ± 0.4
Group 2: 150 mg [^{14}C]caprylic acid			
1.4 ± 0.2	0.6 ± 0.1	5.0 ± 0.3	93.0 ± 0.2
Group 3: 150 mg 2-[^{14}C]ethyl-n-caproic acid			
3.2 ± 1.0	1.2 ± 0.4	47.6 ± 5.1	49.8 ± 5.1

* All emulsions contained 50 mg albumin + 288 mg sodium taurocholate to 3 ml with saline, in addition to the designated fatty acids.

isotope was unabsorbed in 8 h, while about 1% was present in the intestinal wall. These data clearly indicate that the straight-chain acid is more rapidly absorbed in the rat than the 8-carbon branched-chain analogue.

The difference between the radioactivity administered and that recovered (sum of per cent recovered from lymph, small intestine, and combined feces and intestinal contents) gives an approximation of absorption of the fatty acids by a route other than thoracic duct lymph, presumably via portal blood. These data (Table II, apparent portal absorption) show that about 93 and 50%, respectively, of the administered radioactivity appeared in the portal vein blood after the administration of caprylic and 2-ethylcaproic acids. It is of particular interest that these data show that about 11% appeared in portal vein blood when [1-^{14}C]oleic acid was given. The results clearly indicate that a major portion (84.5%) of the labeled oleic acid which left the gut wall was absorbed *via* the lymphatic system. The remainder was transported *via* the portal vein, and as discussed above, this amount was not due to lympho-venous anastomoses.

The rates of appearance of absorbed fatty acids in thoracic duct lymph are shown in Fig. 2. Administration of the aqueous emulsions of ^{14}C-labeled fatty acids to these animals results in a peak of absorption between 2 and 4 h after feeding and a subsequent decline in the appearance of isotope.



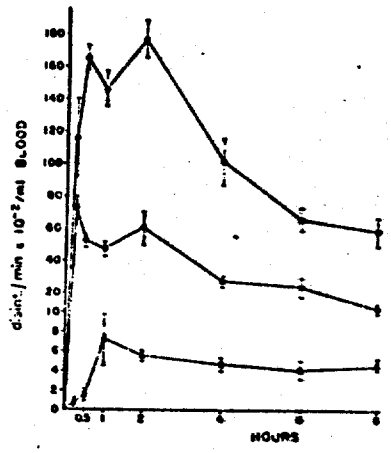


Fig. 2. Rate of appearance of radioactivity in thoracic duct lymph following administration of test emulsions. Emulsion 2: 50 mg albumin + 288 mg sodium taurocholate (control) in addition to 292 mg [^{14}C]oleic acid (4.6 μC); Emulsion 3: Control + 150 mg [^{14}C]caprylic acid (3.0 μC); Emulsion 4: Control + 150 mg 2-[^{14}C]ethyl-*n*-caproic acid (3.2 μC). Values are expressed as per cent of radioactivity recovered in the lymph and represent means of four animals ± S.E. Lymph samples were collected in 2-h periods for 8 h. ▲——▲, [1-^{14}C]oleic acid; ○——○, 2-[^{14}C]ethyl-*n*-caproic acid; ●——●, [1-^{14}C]caprylic acid.

Fig. 3. The appearance of radioactivity in portal blood system following administration of test emulsion. See Table I for composition of test emulsions. Values are expressed as desintegrations/min present per ml of portal vein blood at a given time and represent means of four animals ± S.E. ○——○, 2-[^{14}C]ethyl-*n*-caproic acid; ●——●, [1-^{14}C]caprylic acid; ▲——▲, [1-^{14}C]oleic acid.

Fig. 3 summarizes analysis of portal vein blood after administration of the labeled fatty acids. Since these analyses were carried out on blood from animals with complete thoracic duct lymph drainage, appearance of isotope gives a direct indication of portal transport of fatty acids. With the short-chain acids, caprylic and 2-ethyl-*n*-caproic acid, large amounts of the isotope were present in portal blood within 15 min and the levels reached a peak 15 and 30 min after administration, indicating the very rapid absorption of these acids. The second peak at 2 h may not represent true peaks for portal absorption of these acids. Since by 2 h the blood had circulated several times, radioactivity detected at this time is a combination of the radioactivity already present in the circulation and the radioactivity still being transported from the intestine.

It is also interesting to note that when a similar total dose of radioactivity was given either as [^{14}C]caprylic acid (3.0 μC) or as 2-[^{14}C]ethyl-*n*-caproic acid (3.2 μC), there was two to three times as much ^{14}C-activity present in portal vein blood after 2-ethyl-*n*-caproic acid as after caprylic acid. These high levels of radioactivity in the portal system could be the result of either rapid transport of the branched-chain acid from the intestine to portal vein blood or to slow removal of the absorbed acid from the circulation by other tissues, *i.e.* the liver. Since only about 50% of the fed 2-[^{14}C]-ethyl-*n*-caproic acid was absorbed from the intestine while more than 90% of the fed [^{14}C]caprylic acid was absorbed, the higher levels of radioactivity found in portal blood after the administration of 2-[^{14}C]ethyl-*n*-caproic acid are likely due to the slower clearance of this acid from the general circulation.

The data presented above clearly indicate that short-chain fatty acids, such as

caprylic and 2-ethyl-*n*-caproic acids, are absorbed almost exclusively *via* the portal system (94–98%), and only a small amount (less than 3%) of the fed acid appears in thoracic duct lymph. When [^{14}C]oleic acid was given to lymph and portal vein fistula rats, a major portion of the absorbed acid was recovered in thoracic duct lymph (85%). However, up to 15% of the absorbed oleic acid was transported *via* the portal system. Recently, SAUNDERS AND DAWSON[20] reported that a large proportion of oleic acid is transported *via* the portal system when bile is diverted from the intestine. They suggest that this alteration in the route of transport of absorbed oleic acid in bile-fistula rats may represent a fuller utilization of a minor pathway in normal rats. The data presented in this paper suggest that the portal route plays a significant role in the transport of absorbed long-fatty acids in normal animals. This route of long-chain fatty acid transport may be even more significant when there is a failure in the esterification of these acids in the intestinal mucosa.

Distribution of radioactivity in lipid fractions of thoracic duct lymph and portal vein blood after administration of labeled fatty acids

Distribution of radioactivity in lipid fractions of thoracic duct lymph. After pooling the time samples of thoracic duct lymph, the lymph lipids were extracted and fractionated by silicic acid column chromatography. The per cent distribution of radioactivity in the different fractions is summarized in Table III.

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN LIPID FRACTIONS OF THORACIC DUCT LYMPH

Cholesterol ester (%)	*Triglyceride (%)*	*Minor lipids (%)*	*Free fatty acids (%)*	*Phospholipid (%)*
*Group 1: 292 mg [^{14}C]oleic acid**				
1.1 ± 0.1	85.4 ± 3.1	3.9 ± 1.1	7.8 ± 2.2	1.8 ± 0.3
Group 2: 150 mg [^{14}C]caprylic acid				
0.0	28.7 ± 4.0	2.3 ± 0.7	69.5 ± 4.3	0.0
Group 3: 150 mg 2-[^{14}C]ethyl-n-caproic acid				
0.0	7.9 ± 1.2	1.0 ± 0.3	90.4 ± 1.4	0.0

* All emulsions contained 50 mg albumin + 288 mg sodium taurocholate to 3 ml with saline, in addition to the designated fatty acids. The values represent means ± S.E. of lipid fractions in 8-h samples of lymph.

In Group 1, which received [1-^{14}C]oleic acid, 85% of the radioactivity was present in the form of triglycerides and 2 to 4 % appeared as cholesterol esters and phospholipids. About 8% was present as free fatty acid. This data is comparable to that reported by BORGSTRÖM AND TRYDING[11]. In contrast to [^{14}C]oleic acid, when [^{14}C]caprylic acid or 2-[^{14}C]ethyl-*n*-caproic acid were administered, most of the radioactivity was present as free fatty acid (69.5 and 90.4%, respectively). No radioactivity was present in the cholesterol ester fraction, indicating that neither caprylic acid or 2-ethyl-*n*-caproic acid was directly involved in the formation of lymph cholesterol esters. Furthermore, only 8 to 29% of the radioactivity was in the form of triglyceride. These results with short-chain acids differ from those of BLOMSTRAND[8]

who found that most of the radioactivity recovered in the lymph was present as triglycerides after the administration of [^{14}C]decanoic acid.

In the case of the short-chain fatty acids, the appearance of a small amount of ^{14}C activity in thoracic duct lymph may or may not represent true absorption *via* the lymphatic system. It is possible that the small amount of ^{14}C activity was transferred from the blood to lymph during the course of general circulation. However, it has been reported by BLOMSTRAND[10] that free decanoic acid is not able to pass the blood-lymph barrier and enter the thoracic duct lymph.

Distribution of radioactivity in lipid fractions of portal vein blood. Having established that 12 to 15% of the radioactivity of absorbed [^{14}C]oleic acid is transported *via* the portal system, it was decided to study the distribution of radioactivity in the lipid fractions of portal vein blood after the administration of ^{14}C-labeled fatty acids. Lymph and portal vein fistula rats were given ^{14}C-labeled fatty acids with albumin and sodium taurocholate as in Groups 1–3. (Total doses of ^{14}C activity were 15, 3.0, and 3.2 μC of oleic, caprylic and 2-ethyl-*n*-caproic acids, respectively.) 1 h after the administration of the test emulsions, 2–3 ml of portal vein blood were collected. The blood lipids were then extracted and the lipid fractions were separated by thin-layer silicic acid chromatography[17]. As shown in Table IV, when [^{14}C]oleic acid was administered, 50% of the radioactivity present in portal vein blood was in the form of free fatty acid. However, there were significant amounts of radioactivity present in the monoglyceride, diglyceride, triglyceride and phospholipid fractions. In contrast to [^{14}C]oleic acid, when [^{14}C]caprylic acid was given, essentially all of the radioactivity (96.5–99.9%) was in the form of free fatty acid; only about 2% was present as esterified fatty acid. When 2-[^{14}C]ethyl-*n*-caproic acid was administered, all of the radioactivity present in portal vein blood was in the form of free fatty acid (98.9–102.2%). These results are contrasted to those reported by BORGSTRÖM[11] with [^{14}C]decanoic acid in which 64–74% of the fed ^{14}C activity was present in the form of free fatty acid in portal vein blood.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY PRESENT IN PORTAL BLOOD AFTER ADMINISTRATION OF TEST EMULSIONS CONTAINING ^{14}C-LABELED FATTY ACIDS

The radioactivity was assayed as described in the text. Values are expressed as per cent of total radioactivity recovered in portal vein blood after the administration of ^{14}C-labeled fatty acids. Each fatty acid was administered to two animals.

Lipid fractions	*Received [^{14}C]oleic acid (%)*		*Received [^{14}C]caprylic acid (%)*		*Received 2-[^{14}C]ethyl-n-caproic acid (%)*	
	Rat 1	*Rat 2*	*Rat 3*	*Rat 4*	*Rat 5*	*Rat 6*
Phospholipids	19.1	14.9	0.6	0.3	0.0	0.0
Monoglycerides	9.9	14.4	0.0	0.6	0.0	0.0
Diglycerides	10.7	10.5	0.4	0.4	0.0	0.0
Free fatty acid	49.0	49.6	96.5	99.9	98.9	102.2
Triglycerides	9.9	7.2	1.0	0.7	0.0	0.0
Cholesterol ester	1.3	3.5	0.2	0.3	0.0	0.0

The results obtained in this experiment suggest that long-chain fatty acids and short-chain fatty acids follow different metabolic pathways after they are transported *via* the portal system. 1 h after the administration of [^{14}C]oleic acid, as much as 50% of the radioactivity recovered in portal blood was present as esterified fatty acid.

It is possible that a large portion of the oleic acid which is transported *via* the portal system is taken up by the liver and rapidly esterified. In case of short-chain fatty acids, most of the acid present in the portal system was in the form of free fatty acid, indicating that these acids are not esterified effectively by enzymes present in the liver and other tissues. It is likely that these acids, when transported *via* the portal system, are removed from the circulation, most probably by the liver, and catabolized to carbon dioxide, or are excreted in the urine.

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EFFECT OF FATTY ACIDS ON THE PROTEOLYSIS OF PROTEINS

I. EFFECT OF C_6-C_{14} FATTY ACIDS ON THE TRYPTIC DIGESTION OF OVALBUMIN

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It has been repeatedly demonstrated (*1*) that ovalbumin is easily digestible by enzymes after denaturation by heating, while, it is hardly digestible in the native state. The present author also reported (*2*) some results of her studies on the effects of heating on the tryptic digestibility of ovalbumin. The maximum digestion of egg white and egg albumin by trypsin was obtained by a heat treatment at 80° for 10 minutes (*2, 3*). When food protein is digested in the alimentary canal, it is usually accompanied with fatty acid. To clarify how the digestion of ovalbumin is affected by fatty acid, is interesting with respect to the physiology of digestion or enzymatic degradation of protein. The present study was designed to elucidate the effect of various fatty acids on the digestion of ovalbumin by trypsin.

EXPERIMENTALS AND RESULTS

I. Digestion Test

(*A*) *Materials:*

Crystalline Ovalbumin—Prepared by the modified method of Kekwick and Cannan. (*4*). N-content 14.82 per cent.

Enzyme—Crystalline trypsin kindly supplied from Mochida Seiyaku Co. 1270 unit/mg.

Fatty Acids—Fatty acids used were *n*-caproic, *n*-caprylic, *n*-capric, *n*-lauric and *n*-myristic acids. All the acids were approximately 99 per cent pure.

(*B*) *Experiments:*

Preparation of Test Solution—An amount of 110 mg. of ovalbumin was taken into a 10 ml. conical flask and was dissolved with 2 ml. of water and 3 ml. of 0.3 *M* phosphate buffer (pH 7.0), followed by the addition of a definite amount of Na-salt of fatty acid. The final volume of the solution was made up to 10 ml. with water.

Enzyme Solution—The solution contained 5.5 mg. of crystalline trypsin in 1 ml. of water.

Denatured Ovalbumin Solution—The ovalbumin solutions were heated in a water bath (at 80° and 98°) for 10 minutes.

Tryptic Digestion—Digestion temperature: 37°, Digestion time: 1, 5, 10, 20 and 40 minutes, respectively. Three sets of 5 ml. of ovalbumin solution containing fatty acid were

Cap. acid

treated as follows:

a. control
b. heated at 80° for 10 minutes.
c. heated at 98° for 10 minuees.

Each of them was taken into 20 ml. test tube and kept at 37°. Thereupon, 1 ml. of enzyme solution was added to the test tube and mixed. At different time intervals, namely, after 1, 5, 10, 20 and 40 minutes' incubation, 1 ml. of the reaction mixture was taken out and poured into 10 ml. of 5 per cent trichloracetic solution and the resulted presipitate was filtered off.

The optical density of the filtrate was estimated with a spectrophotometer at 280 mμ, and the digestion coefficient. (TCA soluble "tyrosin"/total "tyrosin" of ovalbumin ×100) was calculated. As for a blanc test, 0.83 ml. of the substrate solution, 0.17 ml. of the engyme solution and 10 ml. of the trichloracetic acid solution were mixed at the same time and filtered. The absorbance of this filtrate was subtracted from the above estimated absorbances.

(C) *Results:*

As shown in Table I, the proteolytic activity of trypsin towards denatured ovalbumin stopped almost completely after 40 minutes incubation under these conditions. The maximum degree of digestion by longer incubation time was approximately 80 per cent. As shown in Table I, C_8 fatty acid does not affect the digestion of ovalbumin by trypsin even at the concentration of 0.4 *M*: C_3 acid, however, accelerates the digestibility of ovalbumin at 0.1 *M* to almost the same degree as the heat denatured ovalbumin, and inhibits the digestion at higher concentrations. The longer the carbon chain of fatty acid, the stronger this effect appears, and C_{12} acid has the effect for accelerating the digestion even at 0.015 *M*, and shows inhibition at 0.05 *M*. The C_{14} acid also shows the same effects, at concentrations ranging from 0.0025 to 0.015 *M*.

II. *Determination of Specific Rotation of Ovalbumin Solution*

In order to recognize whether or not ovalbumin is denatured by the addition of fatty acid, the specific rotation of ovalbumin was determined,

TABLE I

Effect of Fatty Acid Added on the Digestion of Ovalbumin by Trypsin

(a) Control (without fatty acid)

Treatment / Digestion time, (*min.*)	Digestion coefficient (%)		
	Unheated	Heated at 80°	Heated at 98°
5	2.4	26.0	24.1
10	3.5	42.0	28.5
20	4.1	50.3	34.7
40	7.5	59.2	36.7

(b) Fatty acid added

C_6 *n*-caproic acid

Concentration (*M*)	Digestion coefficient (%)											
	0.1			0.2			0.3			0.4		
Treatment / Digestion time, (*min.*)	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	3.2	34.8	29.5	2.5	—	—	2.7	—	—	2.3	—	—
10	5.8	34.3	31.0	6.1	—	—	6.5	—	—	7.5	—	—
20	7.2	43.3	37.5	7.4	—	—	8.2	—	—	7.5	—	—
40	8.1	50.3	41.3	9.2	—	—	7.6	—	—	7.5	—	—

C_8 *n*-caprylic acid

Concentration (*M*)	Digestion coefficient (%)											
	0.05			0.1			0.15			0.8		
Treatment / Digestion time, (*min.*)	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 90°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	2.5	25.2	24.6	26.8	21.9	25.5	26.2	20.3	21.0	2.3	3.5	1.2
10	3.5	38.1	30.0	30.9	33.2	30.5	35.8	31.0	28.3	1.7	2.9	3.8
20	10.8	47.5	38.1	36.0	41.3	40.0	45.1	40.2	34.0	2.0	1.9	3.6
40	8.7	57.0	46.3	40.1	51.0	46.5	50.2	49.9	40.5	0.9	6.1	5.7

C_{10} *n*-capric acid

Concentration (*M*) / Treatment / Digestion time, (*min.*)	Digestion coefficient (%)											
	0.005			0.015			0.05			0.1		
	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	5.2	32.4	24.3	20.2	32.3	28.7	29.4	28.9	28.1	12.4	19.0	10.8
10	6.1	41.4	30.1	22.2	43.4	34.4	39.6	38.6	35.4	15.4	19.5	12.6
20	6.6	58.6	37.8	27.5	55.9	42.1	47.0	42.6	37.6	17.8	21.2	17.0
40	9.1	58.4	44.9	29.9	59.9	47.5	55.0	47.2	42.7	19.2	21.1	20.2

C_{12} *n*-lauric acid

Concentration (*M*) / Treatment / Digestion time, (*min.*)	Digestion coefficient (%)											
	0.0025			0.005			0.015			0.05		
	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	9.4	34.6	27.9	17.5	40.4	29.5	34.3	41.5	37.8	9.4	7.8	10.0
10	13.0	47.0	37.9	24.2	48.4	41.4	40.8	47.5	45.3	14.5	14.5	13.4
20	16.0	56.3	45.7	35.7	57.0	46.6	44.9	50.7	47.5	17.1	16.7	20.3
40	18.9	63.0	48.4	51.0	63.8	52.7	53.5	57.5	53.0	21.8	21.1	23.8

C_{14} *n*-myristic acid

Concentration (*M*)	Digestion coefficient (%)											
							0.015			0.05		

C_{14} *n*-myristic acid

Digestion time, (*min.*) \ Concentration (*M*) / Treatment	Digestion coefficient (%)											
	0.0025			0.005			0.015			0.05		
	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°	Unheated	Heated at 80°	Heated at 98°
5	11.6	39.4	34.0	15.5	39.7	37.1	32.9	50.7	50.2	13.0	13.4	8.4
10	16.7	49.6	44.3	19.9	54.0	51.1	41.2	62.5	63.3	19.5	19.9	18.1
20	26.8	59.9	51.7	22.8	61.8	57.1	44.2	70.4	70.0	24.8	24.2	23.8
40	18.9	66.9	56.2	23.3	64.9	60.4	47.4	76.9	76.7	25.1	26.3	26.5

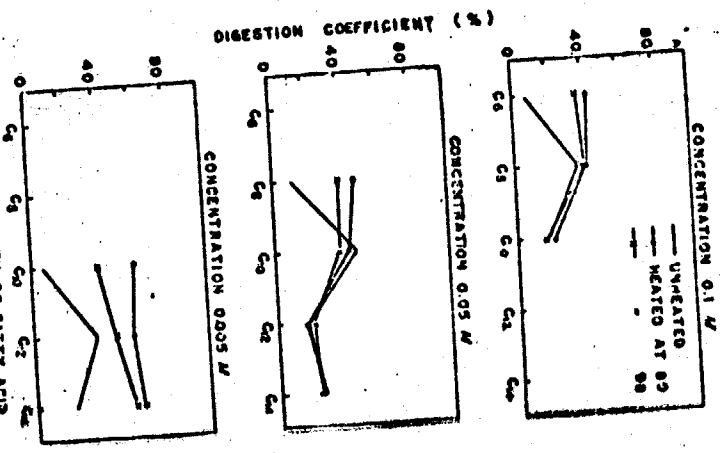

FIG. 1. Effect of fatty acid added at various concentrations on the digestion of ovalbumin by trypsin. Digestion time: 20 minutes.

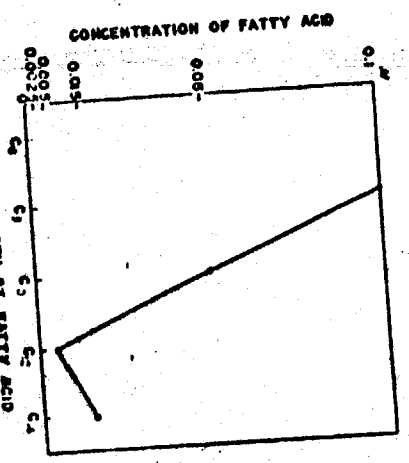

FIG. 2. Concentration of fatty acids at which the digestibility of native ovalbumin by trypsin exceeds 35 per cent. Digestion time: 20 minutes.

because the laevo-rotatory power of protein solution usually increases with its denaturation.

(*A*) *Samples:*

Native Crystalline Ovalbumin (Previously described)—1 per cent ovalbumin phosphate buffer solution (0.1 *M*, pH 7.8).

Fatty Acid Added Ovalbumin—One ml of 2 per cent ovalbumin phosphate buffer solution (0.2 *M*, pH 7.8) was added by proper quantities of fatty acid and 1 ml. of water to give final concentrations of 0.0025 *M*, 0.005 *M*, 0.015 *M* and 0.1 *M* respectively.

(*B*) *Experiments:*

The specific rotation of native ovalbumin (Sample a) was determined in a 5-cm. tube with Lippich's polarimeter at 25°. The fatty acid was added to ovalbumin solution (Sample b), the mixture was incubated 20 minutes at 37° followed by the centrifugation (12,000 r.p.m.) to remove the resulted turbidity. No detectable loss of nitrogen compounds by centrifugation was observed. The specific rotation was determined in the same way as above.

(*C*) *Results:*

It was shown that C_8 fatty acid did not cause any change in the optical rotation of ovalbumin solution even at the concentration of 0.05 *M*, but the rotation increased to laevo at 0.1 *M*. The longer fatty acids caused this

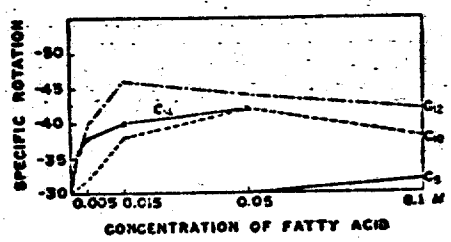

FIG. 3. Change in the optical rotation of ovalbumin by the addition of various fatty acids.

effect at lower concentrations, and C_{12} acid had the strongest effect at the concentration of 0.015 *M*.

DISCUSSION

The results obtained in the experiment I indicated that the addition of fatty acids to ovalbumin solution gave a change like the denaturation by heating, that is, an increase of digestibility by trypsin.

Concerning the effect of surface-active substances, it is reported by P.D. Boyer *et al.* (*5*) that denaturation of serum albumin is inhibited strongly by salts of fatty acids, especially by the acids whose carbon chain consists from 7 or 8 to 12 carbons. Also T. Sato recently reported that both the substrate (*i.e.*, hemoglobin, casein) and trypsin are denatured by sodium laurylsulfate (*6*). Considering these facts, it is apparent that ovalbumin is denatured by fatty acids and becomes more digestible by trypsin. This property of ovalbumin is entirely different from that reported on serum albumin (*6*). The optimum concentration of fatty acids at which tryptic

digestion of ovalbumin is distinctly promoted without heating differs from fatty acid to fatty acid. C_{12} acid had the strongest action among those tested and other fatty acids showed less effect. This is in accord with the fact that the denaturing action of alkylsulfate on the protein is most strong in C_{12} acid (*7*). The tryptic digestion of ovalbumin decreased above a certain concentration of added fatty acid, this might be explained by the assumption that fatty acids denaturate not only ovalbumin, but also trypsin itself as indicated in Sato's experiments. As the experiment II indicates, the optical rotation of ovalbumin solution is changeable with the addition of fatty acid, and the effect has almost the same tendency as on the tryptic digestion, that is, C_{12} acid has the strongest power at the lower concentration among the tested C_8 to C_{11} acids. It is generally believed that the change of optical rotation of the protein solution is caused by the denaturation, (of protein.) for instance by the unfolding of protein molecule (*8*). The results in the experiment I indicates that the denaturations by heating and by added fatty acids are probably the same kind of modification of proteins at least in a sense that both preparations are susceptible to tryptic digestion.

SUMMARY

1. C_8 to C_{11}-fatty acids promote the tryptic digestion of ovalbumin, but C_6-fatty acid does not at concentrations 0.1–0.4 *M*.

2. Ovalbumin becomes susceptible to tryptic digestion by the addition of fatty acids nearly as much as by heat denaturation.

3. At higher concentration of fatty acids the tryptic digestion decreased for both heated and unheated ovalbumin.

4. The optimum concentrations of fatty acids for tryptic digestion of ovalbumin decrease with the increase of the carbon chain.

5. The laevo-rotatory power of ovalbumin solution increases by the addition of fatty acid. The effect differs from fatty acid to fatty acid.

6. The greatest change of optical rotation is obtained by C_{12} acid at 0.015 *M* concentrations, indicating that C_{12} acid is most effective in a modification of protein structure. This fits in with the observation by tryptic digestion.

The author wishes to express her sincere thanks to Prof. S. Akabori and Assist. Prof. B. Maruo for their kind guidance and encouragement throughout this work.

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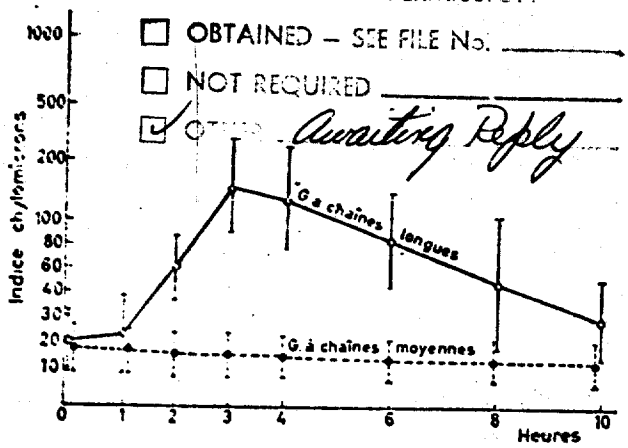

Fig. 4. Courbes d'hyperlipémie (indice-chylomicrons) de 10 sujets normaux après ingestion de 100 g d'huile d'arachide ou d'olive émulsionnée (graisse à chaînes longues) ou de 100 g de graisse synthétique émulsionnée (graisse à chaînes moyennes).

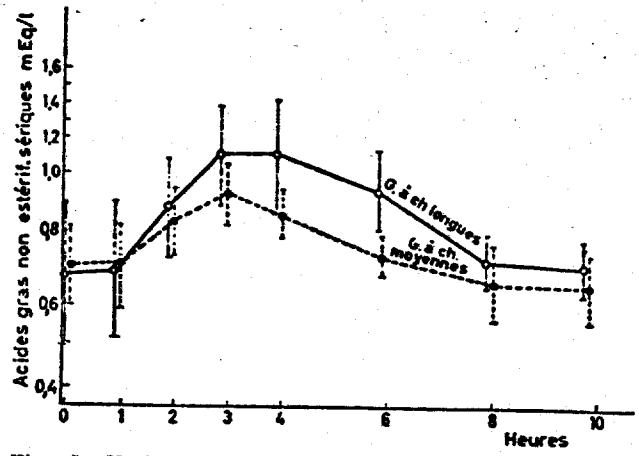

Fig. 5. Variations des acides gras non estérifiés sériques après ingestion de 100 g d'huile d'arachide ou de 100 g de graisse à chaînes moyennes (5 sujets).

Chez un sujet atteint d'une *chylurie* due à une fistule entre le système lymphatique et le bassinet gauche, un régime à base de TCM a arrêté l'élimination de chylomicrons urinaires en quelques jours, mais la chylurie réapparait dès que le sujet est placé à un régime hypergras, à TCL.

Enfin plusieurs *hyperlipémiques* ont été placés pendant plusieurs jours, plusieurs semaines ou plusieurs mois à un régime contenant entre 60 et 100 g de TCM. Leur lipémie est revenue dans tous les cas à la normale, sauf chez un sujet atteint d'une très forte hyperlipémie (120 g ‰) à sérum lactescent, induite par les graisses et les glucides. Chez ce malade, le régime à base de TCM a ramené la lipémie à 40 g ‰, minimum qui n'a jamais pu être dépassé.

Les graisses à acides gras moyens présentent encore d'autres applications diététiques puisque facilement absorbées sans produire de chylomicrons, elles peuvent couvrir les besoins énergétiques, comme tous autres lipides sans en présenter certains inconvénients.

III — Travaux effectués au Centre de Recherche sur la cellule normale et cancéreuse, Villejuif

E. LE BRETON et P. LEMARCHAL :

Catabolisme de l'acide octanoïque par les mitochondries de foie de rat. Comparaison entre le foie normal et le foie stéatosique

On sait qu'un grand nombre de facteurs peuvent donner lieu chez l'homme et l'animal à une accumulation anormale de graisses dans le foie. Parmi ceux-ci, on trouve divers toxiques tels que les dérivés halogénés, le phosphore, l'exposition aux rayons X, ainsi que des facteurs hormonaux et nutritionnels, tels que l'ingestion de trop grandes quantités d'alcool.

La stéatose dont il est question ici sera du type nutritionnel, causée par l'administration d'un régime hypoprotéique et hyperlipidique. D'après E. Le Breton (1954), la carence protéique constitue un « facteur d'agression » susceptible de déclencher une hypersécrétion d'hormone surrénalienne (adrénaline), qui mobilise les graisses de réserve des dépôts (Clément, 1951). Les acides gras, véhiculés par le sang sous forme non estérifiée, sont captés par le foie et stockés dans cet organe sous forme de triglycérides. Si cette réserve lipidique n'est pas l'objet d'une utilisation accrue de la part de l'organe, soit par exportation de lipoprotéines vers le plasma, soit par β oxydation mitochondriale, la stéatose en résulte. On sait d'autre part que l'adjonction de vitamine B_{12} ou de choline dans le régime corrige cette accumulation des triglycérides hépatiques, par un mécanisme non encore complètement élucidé (Mazzei, 1967), d'où le nom de « lipotropes » qui leur a été donné.

Il a donc paru intéressant d'examiner l'un des mécanismes fondamentaux de l'utilisation des triglycérides hépatiques, à savoir le catabolisme mitochondrial *in vitro* des acides gras, comparativement chez des rats normaux ou présentant une stéatose hépatique nutritionnelle.

L'acide octanoïque a été choisi comme substrat, pour des raisons déjà exposées (Mazzei, 1967). L'une des raisons invoquées est l'effet destructeur des acides gras libres à longue chaîne et de leurs thioesters sur les membranes, effet d'autant moins prononcé que l'acide gras est plus court.

D'après des recherches antérieures (Lévy, 1951; Lévy et Legrand, 1953; Artom, 1953; Toury et Lévy, 1967), l'activité des systèmes d'oxydation de l'acide octanoïque mesurée *in vitro* subit une chute importante dans le foie stéatosique, comparé au foie normal. Cependant, la technique manométrique de Warburg utilisée ne permet pas de distinguer nettement l'activité octanoxydasique propre de la « respiration endogène » mitochondriale. On mesure en fait une consommation d'oxygène qui est la résultante du catabolisme de l'acide octanoïque et de l'oxydation de constituants biochimiques apportés avec l'extrait enzymatique lui-même. L'étude de l'oxydation de l'acide octanoïque a donc été faite à l'aide d'une technique plus spécifique en utilisant un substrat marqué par le ^{14}C. Ce sont les résultats de ces recherches qui sont exposés ici.

1. Matériel et méthodes

1) Animaux

Des rats Wistar (souche Commentry) reçoivent dès le sevrage et pendant un mois un régime standard équilibré. Ils sont ensuite divisés en deux groupes, soumis respectivement aux deux régimes suivants : stéatogène (M_2) et témoin équilibré (M_6), décrits sur le tableau I.

2) Incubations

Après un à cinq mois de régime, deux animaux (un de chaque groupe) sont sacrifiés. Une partie du foie est réservée au dosage des lipides. Sur une deuxième fraction, les mitochondries sont préparées suivant Schneider et Hogeboom et mises à incuber dans des conditions voisines de celles utilisées par Lehninger (1948) et par les différents auteurs précités (tableau II). Un marqueur radioactif en $^{8}C(CH_3)$ ou en ^{1}C (COOH) est ajouté au milieu. L'incubation est effectuée dans un tube de centrifugeuse bouché et muni de deux tubulures permettant ultérieurement de récupérer le gaz carbonique par barbotage d'azote.

Dans les conditions d'incubation précédentes, d'après Lehninger et en accord avec nos résultats, l'octanoate est transformé en acétoacétate suivant le schéma :

$$\text{octanoate} + 3O_2 \rightarrow 2 \text{ acétoacétate} + 2H_2O \quad (1)$$

$$C_7H_{15}COOH + 3O_2 \rightarrow 2CH_3 - CO - CH_2 - COOH + 2H_2O.$$

La réaction (1) suit en fonction du temps une loi de linéarité.

Nous avons observé qu'une quantité négligeable de $^{14}CO_2$ est dégagée au cours de la réaction, bien qu'il soit possible, en particulier en augmentant considérablement le rapport $\frac{\text{N mitochondrial}}{\text{substrat}}$, d'obtenir une oxydation plus complète jusqu'à $^{14}CO_2$.

Pour chaque préparation mitochondriale, deux incubations sont effectuées, avec des mitochondries correspondant respectivement à 0,1 g et 0,2 g de foie.

TABLEAU I

Composition des régimes

	Régime M_2 (stéatogène)	Régime M_6 (témoin)
	(%)	(%)
Caséine dévitaminée	8	22
Saccharose	62	58
Saindoux	20	10
Mélange salin	5	5
Mélange vitaminique n° 4 (O choline OB_{12})	2,5	
Mélange vitaminique n° 2 (+ choline + B_{12})		2,5
Agar Agar	2,5	2,5

TABLEAU II

Milieu d'incubation

	µmoles
Tampon phosphate pH 7,4	40
Cl_2Mg	24
ClK	200
ATP	4
Cyt c	0,04
Octanoate de sodium non radioactif	1,6
Octanoate 8 ^{14}C ou 1 ^{14}C	0,02 à 0,05
Mitochondries, quantité correspondant à 0,1 ou 0,2 g de foie initial (1), en suspension dans ClK 11,5 ‰ (1 ml/g de foie).	

250 000 à 615 000 dpm au total dans chaque essai.
Incubation 30 mn à 28 ou 30° avec agitation.

(1) Deux incubations par essai.

3) Mesure de l'activité octanoxydasique

La réaction est arrêtée par addition de 0,5 ml d'acide chlorhydrique normal; le gaz carbonique dégagé est chassé par barbotage d'azote (10 ml/mn), pendant 15 mn à 0°.

On ajoute ensuite au milieu 10,5 ml d'acide trichloracétique à 10 % et 10 ml du mélange éther de pétrole-benzène 1-1 (V/V). Après agitation et centrifugation, il se forme trois phases :

— le précipité protéique;
— la phase aqueuse, contenant les corps cétoniques;
— la phase éthérée, contenant l'acide octanoïque restant après réaction.

Cette dernière est prélevée, et l'on extrait la phase aqueuse encore deux fois par le mélange éther de pétrole-benzène. La phase aqueuse est ensuite débarrassée des dernières traces de solvants organiques par balayage d'azote. Elle est ensuite prélevée, traitée par le bichromate et soumise à distillation selon Weischelbaum et Somogyi (distillation des quatre cinquièmes du volume total soumis à distillation). Nous savons que le bichromate oxyde l'acide β hydroxybutyrique éventuellement présent en acide acétoacétique, et que celui-ci est décarboxylé pour former de l'acétone :

$$CH_3 - CO - CH_2 - COOH \rightarrow CH_3 - CO - CH_3 + CO_2 \quad (2)$$

L'acétone formée est récupérée quantitativement dans le distillat; elle correspond donc à la totalité des corps cétoniques formés au cours de l'incubation. Le distillat est amené à volume connu, ainsi que l'ensemble des phases éthérées provenant de l'opération précédente. Des parties aliquotes sont prélevées pour le comptage de la radioactivité par scintillation liquide.

L'azote mitochondrial est dosé par la méthode de Kjeldahl ou de Nessler.

2. Résultats

1) Mise en évidence de la stéatose

Sur 6 couples de rats étudiés, 5 ont été trouvés porteurs d'un foie stéatosique ainsi qu'il résulte des teneurs en lipides (tableau III) chez certains; l'accumulation de lipides est considérable (surcharge égale à 6 fois la quantité normalement présente), dépendant du temps de régime. Une étude en microscopie électronique a montré la présence de gouttelettes lipidiques réparties au sein du cytoplasme, et même du nucléoplasme, accompagnant une hypertrophie du système de Golgi.

2) Activité octanoxydasique

Le tableau III résume les résultats obtenus, en indiquant, pour chaque préparation mitochondriale, en p. 100 de la radioactivité initiale, la radioactivité restant dans la phase éthérée (octanoate), celle apparue dans la phase aqueuse (corps cétoniques) et celle du $^{14}CO_2$.

On notera, dans le cas d'un substrat $1^{14}C$; que la radioactivité de l'acétone n'a pas été recherchée, puisque la décarboxylation même de l'acétoacétate élimine la radioactivité correspondante sous forme de $^{14}CO_2$.

On peut constater tout d'abord que la radioactivité du gaz carbonique dégagé est négligeable, que l'octanoate initial soit marqué en position 8 ou 1. Les enzymes du cycle de Krebs n'interviennent donc pas dans les conditions de milieu choisies. Dans le cas d'un substrat $8^{14}C$, toute la radioactivité de l'octanoate transformé est retrouvée dans l'acétone du distillat, c'est-à-dire dans les corps cétoniques. Ces faits sont conformes au schéma de la réaction 1, mais n'en constituent pas une preuve absolue, la radioactivité des corps cétoniques n'ayant pu être mesurée dans le cas d'un substrat marqué en position 1.

TABLEAU III

Radioactivités après incubation

(en % de la radioactivité initiale en 30 mn et pour 0,1 g de foie)

N° exp.	Semaines de régime	Régime	Surcharge lipidique mg/g foie	Substrat	Disparue dans l'octanoate	Apparue dans l'acétone	Apparue dans CO_2
1	6	M_2 ...	124	$8^{14}C$	32	30	0,6
		M_6 ...	•	$8^{14}C$	31	27	0,3
2 (28°)	6	M_2 ...	39	$8^{14}C$	21	18	0,8
		M_6 ...	•	$8^{14}C$	22	17	0,6
3	6	M_2 ...	5	$8^{14}C$	35	26	2,5
		M_6 ...	•	$8^{14}C$	36	25	0,5
4	8	M_2 ...	177	$8^{14}C$	15	13	
		M_6 ...	•	$8^{14}C$	17	14	
5	8	M_2 ...	402	$1^{14}C$	26		1,3
		M_6 ...	•	$1^{14}C$	27		0,8
6	22	M_2 ...	370	$1^{14}C$	18		
		M_6 ...	•	$1^{14}C$	48		

Il apparaît clairement par contre, si l'on compare les résultats obtenus avec les foies stéatosiques d'une part, les foies normaux de l'autre, que la vitesse de disparition de l'acide octanoïque, ramenée au gramme de foie frais initial, est la même dans les deux cas.

La seule exception concerne le rat n° 6 (celui qui a été maintenu le plus longtemps au régime stéatogène). En ramenant la vitesse de disparition de l'octanoate au milligramme d'azote mitochondrial, on voit qu'elle est même sensiblement supérieure dans les foies stéatosiques (tableau IV). Ceux-ci, du fait de leur surcharge en graisses, ont en effet une teneur en azote mitochondrial par gramme de foie frais généralement inférieure à celle des foies normaux, ainsi qu'il apparaît sur le tableau IV et en accord avec des résultats antérieurs (Mazzei, 1967).

TABLEAU IV

Vitesses de disparition de l'octanoate

N° exp.	Semaines de régime	Régime	Surcharge lipidique mg/g foie	Azote mitochondrial mg/g foie	Vitesse *(a)*	Vitesse *(b)*
1	6	M_2	124	3,5	170	49
		M_6	0	4,0	165	41
2	6	M_2	39	2,3	112	49
		M_6	0	3,4	117	34
3	6	M_2	5	4,2	186	44
		M_6	0	4,8	192	40
4	8	M_2	177	3,64	80	22
		M_6	0	4,76	91	19
5	8	M_2	402	3,40	138	41
		M_6	0	4,02	144	36
6	22	M_2	370	1,96	99	50,9
		M_6	0	4,02	259	64

(a) En m/μ moles par minute et par g de foie.
(b) En m/μ moles par minute et par mg d'azote mitochondrial.
Chaque valeur est la moyenne de deux mesures.

3. Discussion et conclusion

L'exposé de nos résultats appelle une première remarque. Nous avons admis dans un premier temps que la réaction enzymatique principale intervenant lors des incubations est la transformation de l'octanoate en acétoacétate (ou β hydroxybutyrate). Nous nous sommes appuyés pour cela sur les recherches de Lehninger, et sur l'absence de $^{14}CO_2$ que nous avons nous-mêmes observée. Une confirmation de ce fait par une analyse de la phase éthérée ainsi que de la phase aqueuse par des techniques spécifiques de dosage des acides gras et cétoniques serait en toute rigueur nécessaire. Cette réserve étant formulée, ces résultats sont interprétés de la façon suivante : bien que la stéatose hépatique de nos rats soit très importante (surcharge atteignant 400 mg par gramme de foie), l'activité octanoxydasique de leurs mitochondries, exprimée en terme d'octanoate disparu, par unité de temps et par milligramme d'azote mitochondrial, est supérieure ou égale à celle des mitochondries hépatiques de rats normaux prises dans les mêmes conditions.

Si l'on constate, comme l'ont fait divers auteurs, une diminution de la respiration mitochondriale, en cas de stéatose nutritionnelle, exprimée en terme de consommation d'oxygène, il n'est pas certain, d'après les résultats exposés ici, que cette diminution intéresse le catabolisme de l'acide octanoïque proprement dit. On peut envisager au contraire, au moins dans les premiers temps (un à deux mois de régime), que le jeune animal réagisse à la surcharge hépatique en graisses par une β oxydation légèrement accrue, insuffisante cependant pour compenser l'afflux des acides gras apportés par le courant sanguin. Si la situation de carence protéique se prolonge, on observe que cette surcharge s'accompagne d'une disparition presque totale des dépôts adipeux de réserve, et plus généralement d'un état physiopathologique extrêmement défavorable (amaigrissement sévère, pelage hérissé). Il est possible que, dans ce cas, le métabolisme mitochondrial hépatique se trouve profondément perturbé, et en particulier l'activité β oxydante. Il ne s'agirait cependant là que d'une situation tout à fait exceptionnelle, celle de jeunes animaux en croissance et soumis pendant un temps prolongé à un régime très déséquilibré.

L'impossibilité où nous avons été d'obtenir même un début de stéatose, avec ce même régime mais chez des rats ayant 8 mois ou plus, constitue une bonne illustration de cette hypothèse.

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Discussion sur les acides gras courts et moyens

R. F. — Après lecture du rapport de M. Clément et coll., on peut se poser la question de savoir si les acides gras courts sont bien utilisés par l'organisme comme les autres acides gras classiques, étant donné qu'ils se présentent eux aussi sous forme de glycérides généralement non homogènes.

G. C. — Ces acides gras, sous cette forme, sont tout à fait bien utilisés par l'organisme, aussi bien par l'organisme total comme l'ont montré beaucoup d'expériences antérieures et plus récemment celles du Pr Métais, que ceux qui sont introduits par voie intraveineuse. M. Métais a montré que ces acides gras sont très rapidement oxydés. Nous trouvons par des perfusions hépatiques ou pulmonaires une oxydation, une métabolisation très rapide. On a même l'impression que, lorsqu'on administre des glycérides homogènes d'acides gras courts, comme la trioctanoïne ou les mélanges des glycérides en $C_6 - C_{10}$, que l'oxydation est extrêmement rapide, beaucoup plus que celle de l'huile de coprah par exemple.

R. F. — Est-ce que vous pensez que les glycérides d'acides gras courts et moyens ont un intérêt particulier ?

G. C. — La réponse paraît bien être oui. Les diététiciens ont trouvé des applications pour certains malades.

R. F. — Et pour l'homme normal ?

G. C. — Ces glycérides peuvent toujours couvrir au moins les besoins énergétiques.

R. F. — Mais ont-ils un intérêt particulier ?

E. B. — Ce sont des acides gras qui sont très vite oxydés lorsque l'animal a froid.

G. C. — ... et qui ne sont pas stockés. L'acide laurique est stocké en partie, mais les acides gras à chaîne plus courte ne se retrouvent qu'à l'état de trace dans les tissus de réserve.

E. B. — Ces acides gras sont vraiment utilisés préférentiellement pour lutter contre le froid, ce qui est déjà intéressant.

J. T. — Si vous vous placez sur le plan de la nutrition humaine, ces acides gras prennent la place d'autre chose.

C'est comme l'alcool, dans une certaine mesure. On observe une oxydation préférentielle.

R. F. — Peut-on dire, par ailleurs, qu'ils peuvent avoir des inconvénients spécifiques ?

G. C. — Pour le moment, je ne peux pas vous répondre.

J. T. — Nous sommes ici dans la médecine. Vraisemblablement ces glycérides qui sont absorbés rapidement doivent être agréables à consommer.

R. F. — C'est exact.

J. T. — Ces produits ont l'inconvénient de ceux qui sont oxydés facilement. Le risque de surcharge calorique est facile.

R. F. — Ce sont des produits qui ont les inconvénients de leurs qualités.

G. C. — Ils ne donnent pas lieu à la formation de chylomicrons, ce qui peut être intéressant dans certains cas.

J. T. — A condition bien entendu de ne pas créer de surcharge calorique.

R. F. — Une bonne alimentation doit comporter un mélange d'acides gras. Quel est le pourcentage acceptable d'acides gras courts dans un bon régime ?

E. B. — Leur absorption facile ne permet pas de fixer une limite. Il ne s'agit que d'une source de calories pour l'organisme, ce n'est pas autre chose.

R. F. — Peut-on dire que l'absence de ces acides gras courts et moyens dans l'alimentation semble être sans inconvénients (des populations entières en sont dépourvues), mais que, au contraire, leur utilisation, dans une mesure raisonnable, est sans inconvénient ?

E. B. — C'est un nutriment qui, à mon sens, n'a aucun caractère de nécessité, mais ne présente aucun inconvénient (1).

R. J. — Et qui peut être une arme diététique dans certains cas spéciaux.

R. F. — Je voudrais faire remarquer que la quantité d'acides gras courts et moyens dans la nature est limitée et que le risque d'en abuser se trouve ainsi réduit, du moins en moyenne.

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(1) Des expériences de E. Le Breton et P. Lemarchal (en cours de publication) montrent que même dans le cas d'une *forte stéatose hépatique*, l'acide octanoïque est oxydé avec une vitesse normale, à condition que le régime contienne assez de protéines (6 à 8 %) pour permettre la synthèse des enzymes impliqués. Ce résultat s'oppose à des faits antérieurs et renforce l'idée d'une bonne utilisation de l'acide octanoïque par les mitochondries et pas seulement pour fournir de la chaleur.

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proximately +16%) represents abnormal thyroid enlargement or that the mean thyroid size is greater by 16% in Nithsdale children.

Geographical variations, both national and local, in the incidence in children and adults in the British Isles have been a regular feature of previous careful surveys (Stocks 1928, Erskine 1933, O'Shea 1946, Murray et al. 1948, Hughes et al. 1959, Trotter et al. 1962, Kilpatrick et al. 1963) and the higher prevalences have almost invariably been found in rural areas where other thyroid abnormalities may have a persistently high incidence. Wilkes' (1966) survey in Derbyshire emphasises this.

More than 100 years ago Mitchell (1862) described goitre in the Nithsdale Valley and the condition seemed to be common enough to merit the term "Nith neck". Local physicians still refer to this term in describing thyroid enlargement in this area (Laurie and Drainer 1967). Other small geographical pockets of endemic goitre in Scotland have been described in the last century (Sloan 1883, McKenzie 1899, Ogilvy 1911, McKay 1914–17).

The ætiology of the goitres found in the present study is not known. In a detailed study of 172 pairs of twins, aged from 12 to 70 years (mean, 21·7 years) from the Glasgow area, the ætiological importance of genetic predisposition, autoimmunity, and iodine deficiency as causes of simple goitre were evaluated. None of these factors were found adequately to correlate with the presence of goitre (McGirr 1966, Greig et al. 1967). The only positive conclusion derived from this study was that goitre in young people is related to a non-genetic female endocrine influence. Our results are consistent with this conclusion, goitre being positively related to the age range 11–15 years and to the female sex, in Glasgow and Dumfries where the prevalence of goitre was relatively low.

The selectively greater prevalence of goitre in pubertal females than in pubertal males is likely to be relevant to the persistence of goitre in adulthood, in which the female/male ratio is high. Whether during and after puberty the female endocrine influence is progoitrogenic or the male endocrine influence antigoitrogenic has, however, yet to be determined. The difference is so striking as to make it unlikely that differences in thyroid-hormone requirements and in iodine requirements alone would account for it.

The higher prevalence of goitre in Nithsdale is not associated with a difference in prevalence among females and males up to the age of 15 years. This, together with the possibility that goitre has been more prevalent in this area for more than 100 years notwithstanding population migration, suggests that an extrinsic factor is mainly responsible for the thyroid enlargements in this rural area. The lack of a preponderance of goitre in the Nithsdale female children may favour the hypothesis that any intrinsic goitrogenic endocrine influence present in adolescence has been preceded by and relegated to a subsidiary role because of the life-long effects of an extrinsic or environmental factor.

Comparative studies are now being made to determine whether this excess of goitre prevalence in Nithsdale is due to correctable factors such as iodine-deficiency state (Crooks et al. 1964, Wayne et al. 1964), or of goitrogenic substances in the flora, milk, and water of the region (Clements 1960, Peltola 1960, Kilpatrick and Wilson 1964).

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EFFECTS OF OCTANOIC ACID ON SERUM-LEVELS OF FREE FATTY ACIDS, INSULIN, AND GLUCOSE IN PATIENTS WITH CIRRHOSIS AND IN HEALTHY VOLUNTEERS

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Summary The effects of intraduodenal infusions of glucose and octanoic acid on the serum-levels of glucose, free fatty acids (F.F.A.), and insulin have been determined in four healthy volunteers and in five patients with cirrhosis. Serum-insulin levels rose in both groups during glucose infusions but the rise was significantly greater when octanoic acid was added. This effect did not seem to be mediated by the serum-glucose because the addition of octanoic acid did not affect this level. Serum-F.F.A. levels fell in both groups but the inclusion of octanoic acid had no effect on the rate of fall of serum-F.F.A.

Cap. Acid

Introduction

IMPAIRED glucose tolerance with elevated fatty-acid levels has been described in patients with obesity (Karam et al. 1965), maturity-onset diabetes (Laurell 1956, Bierman et al. 1957, Randle et al. 1963), acromegaly (Karam et al. 1965), and cirrhosis (Reinberg and Lipson 1950, Dole 1956, Stormont et al. 1961). Randle et al. (1963) suggested that the elevated serum-free-fatty-acid (F.F.A.) levels common to all of these diseases may contribute in part to the reduction in glucose tolerance observed. Furthermore, glucose tolerance has been shown to be impaired in healthy individuals when serum-F.F.A. has been raised by a high fat intake followed by intravenous heparin (Schalch and Kipnis 1965). We have investigated hepatic glucose production as a factor in regulating glucose tolerance in the presence of a F.F.A. flux. A rapid increase of fatty acids in the portal circulation was obtained by adding a medium-chain fatty acid (M.C.F.A.) to a duodenal infusion of glucose. M.C.F.A.s were used because they are transported from the gut by the portal venous system (Kiyasu et al. 1952, Playoust and Isselbacher 1964, Greenberger et al. 1966), in contrast to long-chain fatty acids which are absorbed via the lymphatics. Thus the enteral administration of M.C.F.A. gives rise to the portal perfusion of the liver with these fatty acids and results in acute quantitative as well as qualitative changes of the fatty acids in the portal circulation.

Volunteers, Patients, and Methods

Volunteers and Patients

Four healthy male volunteers, aged between 20 and 35, without any family history of diabetes mellitus were used as controls. Diabetes mellitus was excluded by performing an oral glucose tolerance test (100 g.).

Five patients with moderately severe cirrhosis of the liver were investigated. None had a clinical history of diabetes or of glycosuria (table I). We included patients with cirrhosis

TABLE I—CLINICAL DATA ON THE PATIENTS WITH CIRRHOSIS

No.	Age	Sex	Ascites	Varices	Alcohol intake	Serum-levels of: Total bilirubin (mg. per 100 ml.)	Alkaline phosphatase B.L. units	Albumin/globulin (g. per 100 ml.)	Prothrombin-time (%)
1	44	M	+	+	+	0·3	4·0	3·7/2·0	64
2	67	F	+	+	+	0·7	6·5	3·4/3·4	50
3	51	M	+	−	+	1·4	5·7	2·6/3·9	29
4	65	M	−	+	+	1·0	3·1	3·3/4·8	47
5*	48	M	−	−	+	0·8	7·0	2·8/3·3	8·2

* Laennec cirrhosis diagnosed at liver biopsy.
B.L. = Bessey-Lowry.

because we had found significantly higher serum-octanoic-acid levels after intraduodenally infused octanoic acid in such patients than in non-cirrhotic patients (Linscheer, Patterson, Moore, Clermont, Robins, and Chalmers 1966).

Experimental Design

Two glucose infusions were performed in all subjects. Octanoic acid was added to one of the two infusions in random order. Serum glucose, insulin, octanoic acid, and total F.F.A. were determined before, during, and after the infusion procedure. Octanoic acid alone was infused in two cirrhotic patients.

Technique of Duodenal Infusion

A one-lumen tube (diameter 6 mm.) was passed into the proximal duodenum and positioned under fluoroscopic control. After the tube was properly situated, a test solution was infused for 60 minutes at a constant rate of 6 ml. per minute by means of a Holter infusion pump. The order of the infusion

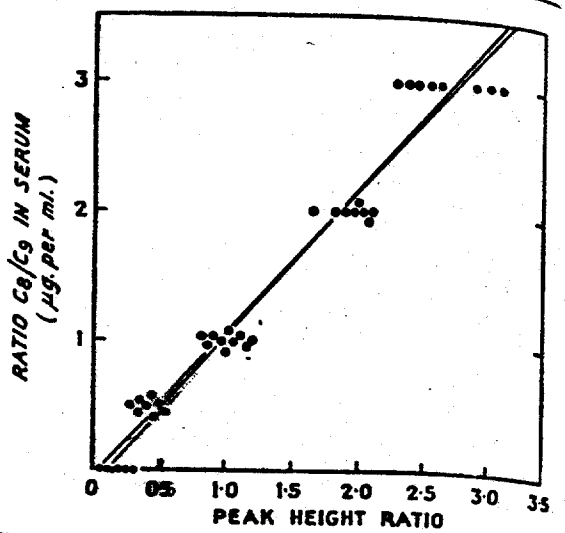

Fig. 1—Regression lines (x on y and y on x) for the ratio of octanoic-acid concentration (0-60 µg. per ml.) to 20 µg. nonanoic acid per ml. and the ratio of the peak heights on the gas/liquid chromatogram. $x = 0{\cdot}0874 + 0{\cdot}8724y$; $y = -0{\cdot}0613 + 1{\cdot}1135x$.

was randomly selected and was performed on different days. At the end of the 60-minute infusion period the tube was removed. Blood-samples for the determination of serum-total-F.F.A., octanoic acid, glucose, and insulin were drawn before, at 10-minute intervals during the 60-minute infusion period, and during the first 2 hours after the removal of the tube (15-minute intervals during the first hour and 30-minute intervals during the second hour).

Infusate

Both test solutions contained glucose (138·9 mg. per ml.), sodium-taurocholate (20 mg. per ml.), and sodium chloride (8·5 mg. per ml.) in water; one solution also contained octanoic acid (10 mg. per ml.).

Analysis of Serum-total-F.F.A.

The Dole method was used when no M.C.F.A.s were present (glucose infusion without octanoic acid). A modification of the Dole method in combination with quantitative gas/liquid chromatography was used when octanoic acid was also present in serum.

Serum (1 ml.) was extracted by 5 ml. isopropanol/heptane/N sulphuric-acid, 4/10/1 (v/v). The heptane layer was then separated by the addition of water (2 ml.) and heptane (3 ml.). The upper phase (3 ml.) was titrated with 0·015 N sodium-hydroxide solution as described by Dole. The long-chain F.F.A. fraction of the serum was completely extracted by this procedure but only a partial extraction (58%) was obtained for octanoic acid. In the absence of octanoic acid a single standard, palmitic acid was used to express the long-chain F.F.A. in µEq. per litre. The serum-F.F.A. is then the product of the nominal value and the ratio of titration equivalents of unknown and palmitic-acid standards. This calculation is invalid if octanoic acid is also present in serum since this acid is incompletely extracted by this method.

The serum-octanoic-acid fraction was measured by quantitative gas/liquid chromatography. The octanoic acid determined by this method is expressed in µEq. per litre and converted into µEq. sodium hydroxide per litre, using the octanoic-acid standard. This is then subtracted from the total amount of sodium hydroxide used for the simultaneous titration of octanoic acid and long-chain F.F.A. extracted from serum (1 ml.). The difference is the long-chain F.F.A. fraction and can be expressed as µEq. per litre using palmitic acid as a standard. Thus the long-chain F.F.A. and octanoic-acid serum fractions are known, and the sum of the two fractions will give a value for the total serum-F.F.A.

Determination of Serum-octanoic-acid by Quantitative Gas/liquid Chromatography

As an internal standard, nonanoic acid (20 µg.) dissolved in acetone (0·04 ml.) was added to each ml. of serum. The serum (1 ml.) was extracted by the Dole procedure (see above). The internal standard allows for correction for incomplete extraction of octanoic acid by the Dole method. After separation of the heptane layer the upper phase (3 ml.) was divided into three portions each. Each portion was evaporated to dryness at 0°C in vacuo by lyophilisation. The dry fatty-acid samples were dissolved in carbon disulphide and a fraction (0·5–1·0 µl.) was used for injection into the gas chromatograph. Octanoic acid in serum was calculated by using the ratios of peak heights (C8/C9) and the ratios of C8 and C9 (C8/C9) in serum (fig. 1). Since C9 in serum is constant (20 µg. per ml.), concentration of C8 can be calculated from the equation:

$$y = 0{\cdot}0613 + 1{\cdot}1135x$$

$$y = \frac{\mu g.\ C8/ml.\ serum}{\mu g.\ C9/ml.\ serum} \text{ and } x = \frac{\text{peak height C8}}{\text{peak height C9}}$$

Standard conditions were vaporiser temperature 260°C, column temperature 180°C, detector temperature 230°C, nitrogen pressure 20 lb. per sq. in. Peaks were identified by retention-time and standards.

Insulin Measurements

Serum-insulin was measured by an immunoassay method (Soeldner and Slone 1965). To test a possible effect of octanoic acid on serum-insulin determinations, octanoic acid (20–200 µEq. per litre) was added to pooled serum, containing 44 µI.U. of insulin. No detectable effect of octanoic acid on the insulin measurements was observed.

Glucose Measurements

True serum-glucose was measured by the glucose-oxidase technique (Washko and Rice 1961).

Statistical Analysis

Since octanoic acid has a high absorption-rate and is metabolised immediately (Schwabe et al. 1964), a statistical analysis was performed on the glucose and insulin data obtained during the infusion period (0–60 minutes). The serum-F.F.A. levels decreased after 10 minutes and the regression lines were calculated for the period between 10 and 80 minutes. Regression lines representing the correlation between serum glucose, insulin, and total F.F.A. concentrations and time were expressed by the formula $y = a + bx$ ($r > 0{\cdot}9$ in all instances). The effect of octanoic acid was measured by determining differences between the slopes of the regression lines and the standard error of the differences.

Results

Serum-glucose Response

In all four volunteers a rise of approximately twice the fasting levels of glucose occurred during the duodenal infusion. A return to preinfusion levels of serum-glucose was reached 45 minutes after the tube had been removed. The addition of octanoic acid to the infusate did not result in significant differences in glucose levels (fig. 2, table II).

In four of the five cirrhotic patients, serum-glucose rose within 10 minutes of starting the infusion. Serum-glucose levels were still elevated 45 minutes after discontinuation of the infusion in all five patients. The addition of octanoic acid to the infusion did not change the serum-glucose concentrations (fig. 2).

Serum-insulin Response

All four volunteers had fasting serum-insulin levels between 2 and 24 µI.U. per ml. A rise in insulin levels concomitant with glucose changes occurred in all cases. In no instance did the serum-insulin exceed 150 µI.U. per ml. (fig. 2). A return to fasting levels of insulin occurred 45 minutes after removal of the duodenal tube in all cases. This corresponded to the return of normal glucose levels. The addition of octanoic acid to a second infusion of glucose resulted in significantly higher insulin levels ($P < 0{\cdot}01$).

Two of the five cirrhotic patients had elevated fasting serum-insulin levels. A rise in serum-insulin occurred in all patients. The maximum rise for each patient did not exceed 160 µI.U. per ml. Addition of octanoic acid to the infusion resulted in peak levels greater than 160 µI.U. per ml. in four of the five patients studied. As in the volunteers, the insulin levels were significantly higher ($P < 0{\cdot}01$)

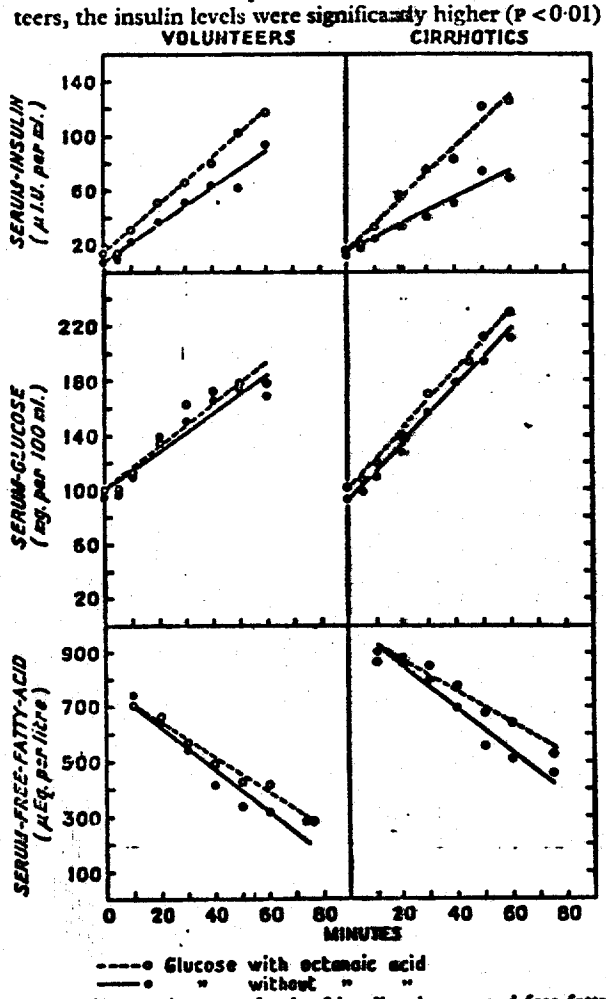

Fig. 2—Changes in serum levels of insulin, glucose, and free fatty acids in controls and in cirrhotic patients receiving glucose with or without octanoic acid.

INSULIN

Controls:
With octanoic acid: $y = 11{\cdot}54 + 1{\cdot}764x$; $r = 0{\cdot}997$.
Without octanoic acid: $y = 7{\cdot}65 + 1{\cdot}330x$; $r = 0{\cdot}981$.

Cirrhotic patients:
With octanoic acid: $y = 15{\cdot}86 + 1{\cdot}479x$; $r = 0{\cdot}962$.
Without octanoic acid: $y = 15{\cdot}72 + 0{\cdot}969x$; $r = 0{\cdot}971$.

GLUCOSE

Controls:
With octanoic acid: $y = 101{\cdot}51 + 1{\cdot}525x$; $r = 0{\cdot}964$.
Without octanoic acid: $y = 99{\cdot}27 + 1{\cdot}435x$; $r = 0{\cdot}959$.

Cirrhotic patients:
With octanoic acid: $y = 97{\cdot}96 + 2{\cdot}259x$; $r = 0{\cdot}996$.
Without octanoic acid: $y = 89{\cdot}07 + 2{\cdot}095x$; $r = 0{\cdot}997$.

FREE FATTY ACID

Controls:
With octanoic acid: $y = 760 - 6{\cdot}356x$; $r = 0{\cdot}992$.
Without octanoic acid: $y = 768 - 7{\cdot}480x$; $r = 0{\cdot}955$.

Cirrhotic patients:
With octanoic acid: $y = 974 - 5{\cdot}676x$; $r = 0{\cdot}979$.
Without octanoic acid: $y = 991 - 7{\cdot}608x$; $r = 0{\cdot}973$.

TABLE II—CHANGES IN SERUM GLUCOSE, INSULIN, TOTAL F.F.A., AND OCTANOIC ACID FROM FASTING LEVELS DURING INTRADUODENAL INFUSION OF GLUCOSE (A) AND GLUCOSE AND OCTANOIC ACID (B)

Serum-level	—	Values and differences at (min.):						
		5	10	20	30	40	50	60
Volunteers (4):								
Glucose (mg. per 100 ml.)	A	5	17	43	56	72	81	75
	B	2	14	36	63	73	79	80
	B–A	3	3	−7	+7	+1	−2	+5
Insulin (μI.U. per ml.)	A	2	14	29	43	56	54	85
	B	−4	13	32	47	61	84	98
	B–A	−2	−1	+3	+4	+5	+30	+13
Total F.F.A. (μEq. per l.)	A	+35	+74	−34	−129	−261	−333	−351
	B	+39	+53	+11	−80	−159	−223	−240
	B–A	+4	−21	+45	+49	+102	+110	−111
Octanoic acid (μEq. per l.).. ..	A	1	1	1	0	0	0	1
	B	+14	+58	+84	130	122	92	96
	B–A	+13	+57	+83	+130	+122	+92	+95
Cirrhotic patients (5):								
Glucose (mg. per 100 ml.)	A	4	15	33	63	85	101	118
	B	5	16	36	68	92	110	126
	B–A	+1	+1	+3	+5	+7	+9	+7
Insulin (μI.U. per ml.)	A	1	16	16	21	34	56	50
	B	5	17	40	58	66	115	110
	B–A	+4	+1	+24	+37	+32	+59	+60
Total F.F.A. (μEq. per l.)	A	50	29	−10	−74	−176	−318	−362
	B	17	24	31	3	−74	−169	−205
	B–A	−33	−5	+41	+77	+102	+149	+157
Octanoic acid (μEq. per l.).. ..	A	1	0	0	1	0	1	0
	B	16	65	145	169	176	193	198
	B–A	+15	+65	+145	+168	+176	+192	+198

when octanoic acid was demonstrable in the systemic circulation. As observed with glucose alone, a delayed return of insulin to preinfusion levels was noted (table II, fig. 2).

Serum-total-F.F.A.

Serum-total-F.F.A. levels fell in all volunteers during the glucose infusion (fig. 2, table II). The lowest level was reached between 15 and 30 minutes after the glucose infusion had stopped and when serum-glucose was receding from the maximal levels reached. 2 hours after ending the infusion the serum-total-F.F.A. had rebounded to fasting levels in all cases. The inclusion of octanoic acid had no significant effect on the rate of fall of the serum-F.F.A. ($P < 0{\cdot}5$).

A fall in serum-total-F.F.A. level occurred in all cirrhotic patients during the glucose infusion. The lowest level was reached between 60 and 90 minutes after the termination of the glucose infusion. The addition of octanoic acid to the glucose infusion did not result in a significant difference ($0{\cdot}10 > P > 0{\cdot}05$) (fig. 2, table II).

Serum-octanoic-acid Response

No measurable levels of octanoic acid were detectable in the four volunteers during the glucose infusion. When octanoic acid was added to the glucose infusion all volunteers showed measurable levels of octanoic acid within 10 minutes of the beginning of the infusion. A maximum concentration was observed within 30 minutes and a return to fasting levels occurred within 45 to 60 minutes after discontinuation of the infusion procedure.

In all five cirrhotic patients no measurable levels of octanoic acid were detected during the glucose infusion alone. Addition of octanoic acid to the infusate resulted in detectable amounts of octanoic acid in the serum within 10 minutes of beginning the infusion. After discontinuation of the infusion, a return of fasting levels of octanoic acid was delayed as compared to the volunteers.

Octanoic Acid Infused Alone by Intraduodenal Tube

Two patients with cirrhosis were investigated. No change in glucose or insulin concentration was detected throughout the infusion. Serum-octanoic-acid levels were observed which were identical with those of cirrhotics given an infusion containing octanoic acid plus glucose.

Discussion

The addition of small amounts of octanoic acid to intraduodenal infusions of glucose (50 mg.) resulted in elevated levels of serum-immunoreactive-insulin as compared with intraduodenal glucose infusions alone. This was true of both healthy volunteers and patients with cirrhosis of the liver. This effect is probably not accounted for by a rise in serum-glucose, since no significant difference in glucose concentrations was observed. It is of interest to note that the serum-insulin rose only during the period of octanoic-acid absorption and it is conceivable that a causal relationship exists.

The finding that the serum-glucose levels remained unchanged during the paired infusion experiments suggests that octanoic acid did not alter the hepatic production of glucose.

Although the slopes of the regression lines of the fatty-acid levels were not significantly different, the fatty-acid levels were probably higher during the octanoic-acid infusion (fig. 2). These slight differences in F.F.A. levels may have been responsible for the observed increase of serum-insulin concentrations, although in the experiments described by Schalch and Kipnis (1965), the glucose tolerance and plasma-insulin levels were affected only by drastic increases in serum-F.F.A.

If the elevation of serum-insulin is not accounted for by the slight rise of F.F.A. during the octanoic-acid infusion, it is possible that octanoic acid exerted a direct stimulatory effect upon the pancreas, or alternatively stimulated the release of an unknown factor in the bowel

all which then affected the release of pancreatic insulin. It is interesting that Horino et al. (1966) have observed a rise in serum-insulin in sheep in response to the intravenous infusion of short-chain fatty acids. This rise also occurred in the presence of unchanged serum-glucose levels.

It has been suggested that the formation of serum-ketoacids, after the administration of the M.C.F.A.s, are responsible for the stimulation of insulin secretion (T'Yangkul et al. 1965). We did not measure serum-ketoacids.

The cirrhotic patients showed significantly higher levels of serum-glucose, insulin, and F.F.A., but the two groups were not age-matched and as glucose tolerance decreases in the older age-groups no conclusions are drawn (Streeten et al. 1965).

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THERAPEUTIC HAZARDS OF PHENYLBUTAZONE AND OXYPHENBUTAZONE IN POLYMYALGIA RHEUMATICA

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POLYMYALGIA RHEUMATICA and temporal arteritis are accepted nowadays as different manifestations of a disseminated arteritis. In most cases of temporal arteritis the diagnosis is obvious at the first examination of the patient. In patients with primarily myalgic symptoms, however, the diagnosis is often obscure for a long time, and the selection of patients for treatment with corticosteroids—the only available treatment that can protect against ocular complications—is difficult. Visual loss is uncommon in myalgic patients, initially free from headache but, according to Olhagen (1963) and Balmforth (1964), this can happen. Thus, the observation of extracranial arteritis in myalgic patients must be of definite clinical importance; patients with suspected polymyalgia rheumatica should be instructed to report immediately to their doctor if they have thickening or pain in the distribution of the temporal artery. We have seen polymyalgic disorders develop into typical temporal arteritis in four patients undergoing therapy with phenylbutazone or oxyphenbutazone. One patient had partial loss of vision in one eye. Though all four patients were completely relieved of their myalgic pains by these drugs, their continued use, by masking progression of the disease, creates a hazard.

The Patients

Case 1

A woman, aged 57 years, with no history of earlier rheumatic disease, had headache and muscular pains in her calves for a short while in 1962. In January, 1963, the pain in her calves was so bad that she could not get out of her bed. She felt very tired and her temperature was slightly raised. The erythrocyte-sedimentation rate (E.S.R.) was 47 mm. in the first hour (Westergren); plasma-fibrinogen 0·8 g. per 100 ml. Oxyphenbutazone was given in a dose of 600 mg. a day for 2 days and then reduced to 300 mg. a day. During the week of treatment, the patient had no muscular pains; her temperature was still raised, however, and her E.S.R. was almost doubled to 91 mm. A temporal-artery biopsy specimen on microscopy was suggestive of arteritis. Prednisone was given instead of oxyphenbutazone, and within a few days the temperature and E.S.R. returned to normal.

Case 2

A man, aged 70 years, was admitted to another hospital in January, 1965, on account of bitemporal headache and fever; his E.S.R., was 100 mm. in the first hour. After 3 days on phenylbutazone 600 mg. a day his temperature was normal and he was completely relieved of his pains. 4 days after he left the hospital he was still free of pain when he noticed a defect of the visual field of his left eye. He returned to the hospital where now a bilateral thickening of his temporal arteries could be detected. Ophthalmoscopy revealed oedema of his left optic disc. The E.S.R. was 129 mm. The patient was sent to our clinic where biopsy of a temporal artery established the diagnosis of arteritis. The E.S.R. decreased rapidly with prednisolone therapy, but the visual-field defect was irreversible. In August, 1965, the treatment was interrupted by mistake; this was rapidly followed by a return of headache, fever, and raised E.S.R. The symptoms again rapidly subsided on reinstitution of steroid therapy.

Case 3

A woman, aged 68 years, was taken ill in August, 1965, with severe pains in the back of her neck, both knee-joints, and left shoulder. She also felt very tired and had lost 12 kg. of weight. The E.S.R. was 80 mm. in the first hour. Her temporal arteries were examined several times without any abnormal findings. All pain disappeared after treatment with oxyphenbutazone 300 mg. a day, subsequently reduced to 100–200 mg. a day without a return of the pain. In October—8 weeks after the therapy was started—we felt circumscribed thickening in relation to the branches of the temporal arteries. Microscopically, there was a typical giant-cell arteritis. The E.S.R. at the time of biopsy was 44 mm. The thickening disappeared with prednisone therapy, and the general condition of the patient improved rapidly.

Case 4

A woman, aged 75 years, was in hospital in July, 1965, on account of haematemesis, probably due to epistaxis. The E.S.R. was 80 mm. in the first hour; this induced a thorough search for malignancy without positive findings. Within a week of her leaving the hospital, she was almost totally immobilised by diffuse myalgic pains over all her extremities and trunk, including the back of her neck. The E.S.R. was 115 mm. Both temporal arteries were normal on palpation. Oxyphenbutazone was given in a dose of 600 mg. a day, reduced after a few days

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STUDIES ON KETONE METABOLISM IN MAN. II. THE EFFECT OF GLUCOSE, INSULIN, CORTISONE AND HYPOGLYCEMIA ON SPLANCHNIC KETONE PRODUCTION[1]

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We have previously described a technique which utilizes hepatic venous catheterization for the estimation of net splanchnic ketone body production (NSKP) in man (1). In a series of subjects without metabolic abnormality, a small but significant endogenous NSKP was detected in the postabsorptive state, and upon the intravenous administration of a ketone body precursor, sodium octanoate, the NSKP was increased fivefold. The present report concerns the application of this technique to the study of the effects of glucose, insulin, cortisone and hypoglycemia on ketone body production in man.

METHODS

Twenty-seven mildly ill male hospital patients between the ages of 23 and 58 years, without metabolic abnormality, were used as subjects and studied after a 12 to 14 hour fast. The criteria of selection and management of subjects, technique of hepatic venous catheterization, methods of chemical analysis, and calculations are identical to those previously reported (1). The 16 normal males in the study will serve as the control series, designated "1955 series," for the present experiments.

The subjects of the present experiments were divided into four groups and studied in a manner similar to the "1955 series" except for the specific metabolic alterations noted below for each group under "Results." The general plan of study for all groups was as follows: After mild phenobarbital sedation, inlying femoral arterial and forearm venous needles and the hepatic venous catheter were inserted. An intravenous infusion of bromsulphalein was started and maintained throughout the period of study. A control study was made on each subject during which femoral arterial and hepatic venous bloods were each sampled four times at five minute intervals for the subsequent determination of bromsulphalein, glucose and total ketone concentrations. A 500 ml. infusion of a 1.5 per cent solution of sodium octanoate was then administered intravenously at a constant rate over a one hour period, during which time blood sampling was repeated four times at 15 minute intervals. Blood sampling for oxygen content and saturation was interspersed at appropriate intervals during each period. The estimated hepatic blood flow (EHBF) was determined by the technique of Bradley, Ingelfinger, Bradley and Curry (2). The net splanchnic glucose production (NSGP) and net splanchnic ketone production (NSKP) are the products of the hepatic blood flow and the hepatic venous-arterial blood glucose and ketone differences, respectively. The net splanchnic oxygen consumption (SPO_2) was calculated by multiplying the arterial-hepatic venous blood oxygen differences by the EHBF.

In addition to the above studies, peripheral venous blood total ketones and glucose concentrations during a four hour insulin tolerance test (0.1 unit per Kg., I.V.) were estimated in 11 normal medical students with and without 200 mg. of cortisone acetate being administered orally one hour prior to the insulin.

RESULTS

1955 series

The data, including the mean plus or minus standard error and standard deviation, for the control series of 16 male subjects previously reported in detail (1), are given at the bottom of Table I. Each subject was studied first in the postabsorptive state, the *control period*, and then during the hour-long infusion of 500 ml. of 1.5 per cent sodium octanoate solution, henceforth designated the *octanoate period*.

Glucose series (Table I)

Six subjects were studied in a manner differing from the 1955 series only in that 20 Gm. of glucose was mixed with the octanoate infusion. Thus, as expected, the data of the control period were entirely comparable to those in the 1955 series of

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TABLE I

The metabolic effects of intravenously administered sodium octanoate mixed with glucose

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.-F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.-F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.-F.A.
yrs. H. M. 37	Functional G.I. 1.71	1+	22.5	30.3	7.8	58.0	80.0	22.0	+35.5	+49.7	+14.2
W. H. 28	Duodenal ulcer 1.59	1+	11.0	15.8	4.8	45.2	48.2	3.0	+34.2	+32.4	− 1.8
J. F. 31	Duodenal ulcer 1.66	1+	14.5	19.2	4.7	52.5	65.2	12.8	+38.0	+46.0	+ 8.1
H. K. 52	Duodenal ulcer 1.91	1+	13.8	15.8	2.0	34.5	51.5	17.0	+20.7	+35.7	+15.0
T. G. 28	Cervical disc 1.77	1+	9.5	11.8	2.3	27.3	36.8	9.5	+17.8	+25.0	+ 7.2
B. U. 30	Functional G.I. 1.98	1+	10.0	16.0	6.0	25.8	41.5	15.7	+15.8	+25.5	+ 9.7
Mean age, 34 Range, 28–52	Mean S.A., 1.77	Mean S.E. S.D.	13.6 1.97 4.83	18.1 2.81 6.89	4.5 0.87 2.14	40.6 5.46 13.39	53.9 6.57 16.09	13.3 2.68 6.57	+27.0 4.05 9.93	+35.8 4.22 10.34	+ 8.8 2.45 6.00
Statistical comparison with control series		p	>0.5	>0.5	>0.5	>0.5	>0.05 <0.1	<0.01	>0.5	>0.05 <0.1	<0.01
Previously reported control series (16 male subjects)		Mean S.E. S.D.	14.1 1.73 6.92	18.4 2.24 8.96	4.3 0.62 2.46	42.6 2.56 10.25	64.8 2.94 11.79	22.2 1.10 4.40	+28.5 2.26 9.01	+46.4 2.83 11.31	+17.9 1.19 4.77

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs. H. M. 37	Functional G.I. 1.71	1+	808	828	+ 20	62	182	+120	34	39	+ 5
W. H. 28	Duodenal ulcer 1.59	1+	548	556	+ 8	28	17	− 11	33	40	+ 7
J. F. 31	Duodenal ulcer 1.66	1+	740	751	+ 11	35	96	+ 61	29	32	+ 3
H. K. 52	Duodenal ulcer 1.91	1+	694	548	−146	16	88	+ 72	28	29	+ 1

TABLE 1—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
T. G. 28	Cervical disc 1.77	1+	1,106	1,238	+132	28	126	+ 98	36	42	+ 6
B. U. 30	Functional G.I. 1.98	1+	764	819	+ 55	46	129	+ 83	35	46	+11
Mean age, 34	Mean	Mean	777	790	+ 13	35.8	106.3	70.5	32.5	38	+ 5.5
Range, 28–52	S.A., 1.77	S.E.	75.3	102.9	31.6	6.60	22.4	18.34	1.35	2.59	1.41
		S.D.	184.0	252.1	84.8	16.2	54.8	44.9	3.31	6.36	3.46
Statistical comparison with control series		p	>0.5	>0.3 <0.4	>0.1 <0.2	>0.5	<0.01	>0.01 <0.02	>0.5	>0.4 <0.5	>0.5
Previously reported control series (16 male subjects)		Mean	790	732	− 58.0	34.0	158.0	+124	36.1	40.3	+ 4.2
		S.E.	38.0	40.1	27.15	5.20	6.86	7.36	2.05	1.52	1.46
		S.D.	151.7	160.4	108.6	20.86	27.46	41.4	7.94	5.83	5.66

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
H. M. 37	Functional G.I. 1.71	1+	14	89	149	+60	50	−30	− 80	94	94	0
W. H. 28	Duodenal ulcer 1.59	1+	0	81	149	+68	62	− 8	− 70	85	83	−2
J. F. 31	Duodenal ulcer 1.66	1+	7	89	140	+51	65	−10	− 75	90	84	−6
H. K. 52	Duodenal ulcer 1.91	1+	10	87	131	+44	72	4	− 68	99	93	−6
T. G. 28	Cervical disc 1.77	1+	12	94	160	+66	96	−41	−137	86	78	−8
B. U. 30	Functional G.I. 1.98	1+	11	81	128	+47	64	−10	− 74	100	103	+3
Mean age, 34	Mean	Mean	9.0	86.6	143	56.4	68.2	−15.8	− 84	92.3	89.2	−3.1
Range, 28–52	S.A., 1.77	S.E.	2.02	2.04	4.96	4.12	6.28	6.72	10.73	2.61	3.74	1.73
		S.D.	4.96	5.00	12.15	10.1	15.40	16.50	26.30	6.40	9.16	4.23
Statistical comparison with control series		p	<0.01	>0.5	<0.01	<0.01	>0.5	<0.01	<0.01	>0.1 <0.2	>0.2 <0.3	>0.5
Previously reported control series (16 male subjects)		Mean	14.9	84.6	81.6	− 3.0	68.0	65.0	− 3.0	84.4	82.2	−3.4
		S.E.	0.88	1.87	1.76	0.5	4.47	6.0	5.88	3.19	3.32	0.73
		S.D.	3.46	7.46	7.05	2.01	17.92	24.08	21.82	12.81	13.01	2.85

TABLE II

The metabolic effect of intravenously administered sodium octanoate mixed with glucose and insulin

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.–F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.–F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs.											
C. M. 39	Cluster headache 1.72	2+	11.0	11.2	0.2	26.7	37.3	10.6	+15.7	+26.1	+10.4
A. H. 28	Duodenal ulcer 1.92	2+	13.5	17.8	4.3	33.0	44.0	11.0	+19.5	+26.2	+ 6.7
A. W. 41	Low back pain 1.76	1+	9.5	12.0	2.5	30.0	48.0	18.0	+20.5	+36.0	+15.5
P. S. 47	Peptic ulcer 1.78	2+	11.0	17.2	6.2	34.0	58.2	24.2	+23.0	+41.0	+18.0
G. F. 33	Epilepsy 1.84	1+	4.2	6.5	2.3	22.0	35.0	13.0	+17.8	+28.5	+10.7
J. G. 30	Peptic ulcer 1.87	1+	12.0	14.7	2.7	31.8	48.5	16.7	+19.8	+33.8	+14.0
C. L. 23	Arrested coccidio-mycosis 1.92	1+	8.8	14.0	5.2	24.5	38.2	13.7	+15.7	+24.2	+ 8.5
E. E. 31	Duodenal ulcer 1.75	1+	36.2	50.8	14.6	52.0	79.5	27.5	+15.8	+28.7	+12.9
Mean age, 34	Mean S.A., 1.82	Mean	13.3	18.0	4.7	31.8	48.6	16.8	+18.5	+30.6	+12.1
		S.E.	3.41	4.85	1.54	3.25	5.15	2.18	1.04	1.98	1.31
Range, 23–47		S.D.	9.66	13.72	4.37	9.20	14.58	6.16	2.94	5.61	3.72
Statistical comparison with control series			>0.5	>0.5	>0.5	>0.02 <0.05	<0.01	>0.02 <0.05	<0.01	<0.01	<0.01

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
C. M. 39	Cluster headache 1.72	2+	731	766	+ 35	3	81	+ 78	34	42	+ 8
A. H. 28	Duodenal ulcer 1.92	2+	1,088	1,147	+ 59	46	132	+ 86	31	38	+ 7
A. W. 41	Low back pain 1.76	1+	623	574	− 49	16	100	+ 84	33	33	0
P. S. 47	Peptic ulcer 1.78	2+	820	689	−131	52	167	+115	33	34	+ 1

TABLE II—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²* Control	During sod. oct.	Δ	Net splanchnic ketone production *μM acetone/min./M.²* Control	During sod. oct.	Δ	Net splanchnic oxygen consumption *ml./min./M.²* Control	During sod. oct.	Δ
yrs.											
G. F. 33	Epilepsy 1.84	1+	627	632	+ 5	14	82	+ 68	28	38	+10
J. G. 30	Peptic ulcer 1.87	1+	631	633	+ 2	18	106	+ 88	25	34	+ 9
C. L. 23	Arrested coccidio-mycosis 1.92	1+	678	775	+ 97	34	108	+ 74	38	43	+ 5
E. E. 31	Duodenal ulcer 1.75	1+	763	815	+ 52	110	223	+113	37	45	+ 8
Mean age, 34	Mean S.A., 1.82	Mean S.E.	745	754	+ 9	36.6	125.0	+ 88.4	32.4	38.3	+ 5.9
Range, 23–47		S.D.	54.8 155.0	63.25 179.0	23.70 67.09	12.06 34.12	17.14 48.52	6.07 17.19	1.54 4.36	1.59 4.49	1.31 3.73
Statistical comparison with control series			>0.5	>0.5	>0.1 <0.2	>0.5	>0.02 <0.05	<0.01	>0.2 <0.3	>0.3 <0.4	>0.4 <0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood* Control	During sod. oct.	Δ	Net splanchnic glucose production *mg./min./M.²* Control	During sod. oct.	Δ	BSP clearance *%* Control	During sod. oct.	Δ
yrs.			*%*									
C. M. 39	Cluster headache 1.72	2+	9	80	106	+26	55	− 7	− 62	79	72	−7
A. H. 28	Duodenal ulcer 1.92	2+	11	86	113	+27	73	+13	− 60	96	91	−5
A. W. 41	Low back pain 1.76	1+	10	82	93	+11	−1	− 2	− 1	70	61	−9
P. S. 47	Peptic ulcer 1.78	2+	14	80	102	+22	58	+ 4	− 54	91	90	−1
G. F. 33	Epilepsy 1.84	1+	8	82	94	+12	91	16	− 75	84	84	0
J. G. 30	Peptic ulcer 1.87	1+	11	82	111	+29	136	+ 2	−134	74	65	−9
C. L. 23	Arrested coccidio-mycosis 1.92	1+	9	79	111	+32	55	0	− 55	79	79	0
E. E. 31	Duodenal ulcer 1.75	1+	13	77	122	+45	56	0	− 56	86	85	−1
Mean age, 34	Mean S.A., 1.82	Mean S.E.	10.7	80.9	106.5	+25.6	65.4	3.4	− 62.0	82.4	78.4	−4.0
Range, 23–47		S.D.	0.66 1.88	0.99 2.80	3.49 9.89	3.87 10.95	13.66 38.66	2.67 7.56	12.86 36.38	3.30 9.35	3.99 11.29	1.40 3.96
Statistical comparison with control series			<0.01	>0.1 <0.2	<0.01	<0.01	>0.5	<0.01	<0.01	>0.5	>0.5	>0.5

TABLE III

Total blood ketone and sugar levels during insulin tolerance tests (0.1 unit insulin per Kg. body weight) in 11 normal medical students with and without cortisone pretreatment

		Total blood ketones μM acetone/100 ml. blood						Blood sugar mg./100 ml. blood					
		Fasting	Δ ½ hr.	Δ 1 hr.	Δ 2 hrs.	Δ 3 hrs.	Δ 4 hrs.	Fasting	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs
Control study	Mean	9.3	−2.4	−2.4	+3.8	+12.9	+16.2	88.0	35.6	67.1	85.4	86.6	85.6
	S.E.	1.38	1.20	1.20	1.72	2.75	3.44	1.66	2.76	3.90	1.90	1.61	1.61
	S.D.	4.47	3.96	3.96	5.50	8.94	11.70	5.51	9.16	12.95	6.30	5.34	5.34
Statistical comparison with fasting levels	p		>0.05 <0.1	>0.05 <0.1	>0.05 <0.1	<0.01	<0.01						
After cortisone	Mean	8.0	−2.4	−0.9	+1.0	+10.7	+12.9	88.7	33.1	70.1	90.4	99.2	99.4
	S.E.	1.38	0.86	1.38	0.86	2.41	3.27	1.46	2.20	4.49	2.44	1.72	1.96
	S.D.	4.30	2.92	4.30	2.75	8.08	11.01	4.84	7.31	14.91	8.12	5.72	6.50
Statistical comparison with fasting level	p		>0.01 <0.02	>0.5	>0.2 <0.3	<0.01	<0.01						
Statistical comparison with control study (p)		>0.5	>0.5	>0.3 <0.4	>0.1 <0.2	>0.5	>0.5	>0.5	>0.4 <0.5	>0.5	>0.1 <0.2	<0.01	<0.01

subjects. After the glucose-octanoate infusion, there was the anticipated elevation of the mean femoral arterial blood glucose level to 143 ± 4.96 (S.E.) mg. per cent and a complete suppression of net splanchnic glucose production. The data on ketone body metabolism in the octanoate period differed from those in the corresponding period in the 1955 series in the following ways; *1*) There was a significant decrease ($p < 0.01$) in the hepatic venous-femoral arterial (HV-FA) blood ketone difference, which was 13.3 ± 2.68 μM per cent compared to 22.2 ± 1.10 μM per cent in the 1955 series; *2*) the mean NSKP was significantly lower ($p < 0.01$) than in the 1955 series, being 106.3 ± 22.4 and 158.0 ± 6.86 μM per minute per $M.^2$, respectively; and *3*) the per cent of infused octanoate estimated to be converted to ketone bodies was 9.0 ± 2.02 per cent, a value significantly less ($p < 0.01$) than the 14.9 ± 0.88 per cent found in the 1955 series. In one subject, H.M., the HV–FA blood ketone difference, NSKP and per cent conversion were not significantly different from the 1955 series. In all other subjects the differences were distinct. There were no significant differences in EHBF, SPO_2 or BSP clearance.

Glucose-insulin series (Table II)

Eight subjects were studied before and after the infusion of 20 Gm. of glucose plus 0.1 unit per Kg. body weight HGF-free insulin mixed with sodium octanoate, bringing out only minor differences from the glucose series. Again, the changes were limited to the octanoate infusion period. During the latter, arterial blood glucose levels were lower than those in the glucose series, but NSGP was equally effectively suppressed. Compared to the 1955 series, there were significantly smaller increases in mean arterial and hepatic-venous blood ketone levels and HV–FA blood ketone differences after octanoate infusion in this group ($p < 0.05$, < 0.01 and < 0.05). Similarly, the octanoate infusion induced a smaller net rise in NSKP than in the 1955 series, mean Δ NSKP being + 88.4 ± 6.07 and + 124 ± 7.36 μM per minute per $M.^2$, respectively ($p < 0.01$), and the estimated per cent conversion of octanoate to ketone bodies was reduced from 14.9 ± 0.88 per cent to 10.7 ± 0.66 per cent ($p < 0.01$). Although there was no significant difference in the octanoate-period NSKP between this and the glucose series, arterial blood ketone levels were lower in the insulin-glucose group ($p < 0.05$). Indirectly this might suggest that insulin promotes the pe-

ripheral utilization of ketone bodies in the presence of a large carbohydrate load, a concept in distinct contrast to the older observations of Chaikoff and Soskin (3) and Mirsky and Broh-Kahn (4) that neither glucose nor insulin affects the utilization of injected ketone bodies in the eviscerate animal.

While the mean NSKP in this group was significantly lower than that of the 1955 series in the octanoate period, two subjects, P.S. and E.E., attained a net rise in NSKP after octanoate infusion that was comparable to that seen in the 1955 series. In Subject E.E., however, the basal NSKP was approximately three times greater than that in the 1955 subjects. This finding, in the absence of starvation or alkalosis, might reflect the presence of greater anxiety, a potential ketogenic stimulus, than was recognized clinically. In Subject P.S., no explanation is apparent from the data.

Insulin tolerance tests in normal medical students (Table III)

Prior to the hepatic venous catheterization studies reported below in the "Hypoglycemic Series," a pilot study of blood sugar and ketone levels during a standard insulin tolerance test was carried out in 11 normal medical students after an overnight fast.

On the control day, marked hypoglycemia was attained in each subject, with a mean blood sugar level of 35.6 ± 2.76 mg. per cent one-half hour after the insulin. The total peripheral venous blood ketone concentrations showed no significant change from the fasting levels until three and four hours after insulin when there was a net rise of 12.9 ± 2.75 and 16.2 ± 3.44 μM per cent, respectively ($p < 0.01$ for both).[6]

Three days later, the tests were repeated one hour after 200 mg. of cortisone acetate had been administered orally. The degree of hypoglycemia at one-half hour and the rise in venous blood ketone levels during the third and fourth hours were not significantly different from the control day. Blood sugar values three and four hours after insulin (four to five hours after cortisone) rose to significantly higher levels ($p < 0.01$) than did those on the control day. On both the control and cortisone treatment days, there was a tendency for blood ketone concentrations to fall one-half hour after insulin, but this was statistically significant only on the cortisone day ($p < 0.02$). Cortisone pretreatment, in this dosage and timing, did not inhibit the rise in blood ketones during the third and fourth hours after insulin hypoglycemia.

Posthypoglycemic series (Table IV, Figure 1)

Brisk but brief insulin hypoglycemia was induced in six subjects at 7 a.m. by the rapid intravenous injection of 0.1 unit HGF-free insulin per Kg. body weight. Three hours later, at 10 a.m., each was studied in the same manner as the 1955 series before and during the infusion of sodium octanoate. Definite hypoglycemia, approximately 30 minutes in duration, was established in all subjects, the mean lowest blood sugar for the group being 31.0 ± 2.46 (S.E.) mg. per cent.

At the time of study by hepatic venous catheterization, the femoral arterial blood glucose levels and the NSGP had returned to normal control levels, but there were striking changes in ketone metabolism. There were significant increases in the arterial and hepatic venous blood ketone levels and a widening of the HV–FA blood ketone differences, the mean values being 38.8 ± 6.2, 57.6 ± 10.70 and 18.8 ± 5.30 μM per cent, compared to 14.1 ± 1.73, 18.4 ± 2.24 and 4.3 ± 0.62 μM per cent, respectively, in the 1955 series ($p < 0.01$, < 0.01 and < 0.05). Mean control period NSKP was 4.7 times greater than that in the 1955 series of subjects ($p < 0.01$). This is shown graphically in Figure 1. It is interesting to note that mean basal NSKP in the posthypoglycemic subject, 160.7 ± 46 μM per minute per $M.^2$, is almost identical with the mean octanoate period NSKP in the 1955 subjects, 158.0 ± 6.86 μM per minute per $M.^2$. If one could assume that the majority of new ketone bodies produced in the posthypoglycemic state arose from endogenous fatty acids, this might yield a rough estimation of the equivalent quantity of fatty acids being converted to ketones in this state. Despite the elevated basal ketone body levels, this group demonstrated approximately the same increase in blood ketone values

[6] In another group of medical students receiving norepinephrine I.V., there was a rise in total blood ketones of [illegible] μM per 100 ml. at the end of three hours. This rise was significantly less than that of the hypoglycemic groups ($p < 0.01$).

TABLE IV

Metabolic studies three hours after insulin hypoglycemia

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: Δ	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: Δ	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs. W. C. 42	Duodenal ulcer 1.77	2+	46.0	60.0	14.0	87.5	125.8	38.3[1]	+41.5	+65.8	+24.3
L. G. 58	Duodenal ulcer 1.71	2+	32.0	44.8	12.8	69.0	88.5	19.5	+37.0	+43.7	+ 6.7
G. K. 34	Duodenal ulcer 1.91	1+	45.5	71.5	26.0	80.8	112.5	31.7	+35.3	+41.0	+ 5.7
O. R. 30	Acne vulgaris 1.68	2+	51.8	92.8	41.0	76.5	128.5	52.0	+24.7	+35.7	+11.0
A. B. 33	Functional G.I. 1.99	1+	46.0	61.8	15.8	67.8	98.2	30.4	+21.8	+36.4	+14.6
H. H. 33	Asthma 1.49	1+	11.2	14.8	3.6	42.0	71.0	29.0	+30.8	+56.2	+25.4
Mean age, 38	Mean	Mean	38.8	57.6	18.8	70.6	104.1	33.5	+31.8	+46.5	+14.7
	S.A., 1.76	S.E.	6.12	10.70	5.30	6.40	9.1	4.4	3.08	4.9	3.49
Range, 30–58		S.D.	15.0	26.30	13.0	15.82	22.4	10.9	7.55	12.0	8.49
Statistical comparison with control series		p	<0.01	<0.01	>0.02 <0.05	<0.01	<0.01	<0.01	>0.4 <0.5	>0.5	>0.1 <0.2

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs. W. C. 42	Duodenal ulcer 1.77	2+	903	866	−37	126	330	+204	30	32	+2
L. G. 58	Duodenal ulcer 1.71	2+	868	790	−78	111	154	+ 43	36	44	+8
G. K. 34	Duodenal ulcer 1.91	1+	914	826	−88	238	261	+ 23	44	37	−7
O. R. 30	Acne vulgaris 1.68	2+	836	825	−11	345	441	+ 96	43	43	0
A. B. 33	Functional G.I. 1.99	1+	749	801	+52	118	230	+112	36	42	+6
H. H. 33	Asthma 1.49	1+	716	644	−72	26	182	+156	27	28	+1

TABLE IV—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
Mean age, 38	Mean S.A., 1.76	Mean S.E. S.D.	831 33.4 81.8	792 31.5 77.1	−39 21.63 53.0	160.7 46.0 112.8	266.3 43.5 106.5	+105.6 27.8 68.0	36 2.70 6.60	37.7 2.65 6.50	+1.70 2.12 5.20
Range, 30–58											
Statistical comparison with control series		p	>0.1 <0.2	>0.1 <0.2	>0.5	<0.01	<0.01	>0.5	>0.5	>0.3 <0.4	>0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
V. C. 42	Duodenal ulcer 1.77	2+	24	82	74	−8	61	43	−18	80	81	+1
[illegible]	Duodenal ulcer 1.71	2+	5	85	80	−5	67	47	−20	97	97	0
G. K. 34	Duodenal ulcer 1.91	1+	3	97	91	−6	64	55	− 9	108	108	0
O. R. 30	Acne vulgaris 1.68	2+	11	94	88	−6	119	122	+ 3	90	89	−1
A. B. 33	Functional G.I. 1.99	1+	15	76	75	−1	73	60	−13	79	75	−5
L. H. 33	Asthma 1.49	1+	15	89	81	−8	50	49	− 1	73	70	−3
Mean age, 38	Mean S.A., 1.76	Mean S.E. S.D.	12.2 3.13 7.68	87.1 3.17 7.76	81.6 2.75 6.74	−5.5 1.10 2.69	72.3 9.80 24.10	62.7 12.1 29.7	− 9.6 3.76 9.22	87.8 5.34 13.08	86.7 5.82 14.25	−1.1 0.91 2.24
Range, 30–58												
Statistical comparison with control series		p	>0.2 <0.3	>0.5	>0.5	0.1	>0.5	>0.5	>0.5	>0.5	>0.4 <0.5	>0.05 <0.1

and NSKP as the 1955 series during the octanoate infusion. In both the control and octanoate periods, mean EHBF, SPO_2 and BSP clearance did not differ significantly from the 1955 subjects.

Cortisone-hypoglycemic series (Table V)

Seven subjects were given 300 mg. cortisone acetate orally at 5 a.m. on the experimental day. Subsequently, they were made hypoglycemic at 7 a.m. and were studied in a manner exactly similar to the "posthypoglycemic series" above. Hypoglycemia was also attained in each of these subjects, the mean lowest blood sugar level for the group being 32.7 ± 2.81 mg. per cent. Due to technical difficulties only a "control period" study was made in Subject D.M.

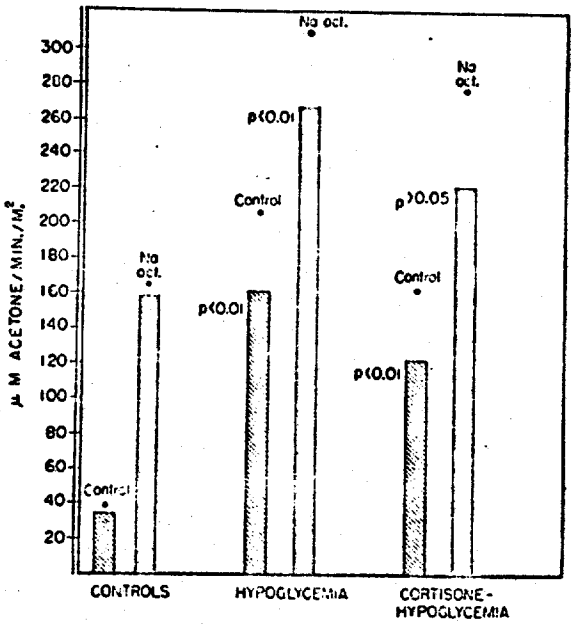

FIG. 1. NET SPLANCHNIC KETONE PRODUCTION BEFORE AND DURING SODIUM OCTANOATE INFUSION IN CONTROL, POSTHYPOGLYCEMIC AND CORTISONE-HYPOGLYCEMIC SERIES OF SUBJECTS (MEAN PLUS OR MINUS STANDARD ERROR)

The p values indicate the statistical comparison between the experimental and control series.

Compared to the posthypoglycemic group, cortisone administered two hours prior to the induction of hypoglycemia did not significantly alter the mean value of any of the measured parameters of carbohydrate or ketone body metabolism. However, closer examination of the ketone data in Table V reveals a remarkable variation of blood ketone levels and NSKP in the individual subjects included in this group. Although comparable degrees of hypoglycemia were attained in each of the seven individuals, Subjects C.P., E.K. and C.F. had the low basal NSKP levels of 37, 41 and 18 μM per minute per M.², as compared to the mean of 160.7 ± 46 in the posthypoglycemic group. On the other hand, Subjects E.H. and L.W. varied in the opposite direction with basal NSKP of 299 and 221 μM per minute per M.². Blood ketone levels revealed a parallel individual variation. Thus, although the mean data in this group indicate that cortisone pretreatment does not affect the ketogenic response to hypoglycemia, the considerable individual variation makes one accept this interpretation with caution.

This group did differ significantly from both the 1955 and posthypoglycemic series in one important respect, the presence of an elevated EHBF in the control period. The mean EHBF in this group was 1,019 ± 71.92 ml. per minute per M.², compared with 790 ± 38 in the 1955 series and 831 ± 33.4 per minute per M.² in the posthypoglycemic series ($p < 0.01$ and < 0.05). This finding is considered to be a direct or indirect effect of cortisone and is consistent with the previously reported data of Myers and Taylor demonstrating a 43 per cent increase in EHBF following cortisone therapy (5). This effect was partially obscured during the octanoate infusion when there was a fall in EHBF in all three groups.

DISCUSSION

In analyzing the data obtained by this technique it must be kept in mind that we are estimating net *splanchnic* ketone body production. While the viscera and mesentery drained by the portal vein are not the sources of ketone bodies *per se*, the mesenteric adipose tissue unquestionably contributes ketone body precursors as nonesterified fatty acids to the liver. Thus changes in NSKP as herein defined may be determined both by alterations in adipose tissue fatty acid release as well as true hepatic ketogenesis.

The data show that glucose added to an intravenous octanoate infusion significantly reduces the splanchnic ketone production observed when the latter alone is infused in man. The addition of insulin to the glucose-octanoate infusion did not alter the magnitude of this response. This effect of glucose is consistent with the large body of experimental evidence, recently reviewed in detail by Campbell and Best (6), suggesting that carbohydrate exerts its antiketogenic effect by suppressing ketone body production by the liver rather than by influencing peripheral tissue utilization of ketone bodies.

From our data it cannot be determined whether glucose affected the utilization of administered octanoate or simply inhibited endogenous ketone production from fatty acids derived from the liver itself or entering the liver from other sources. Observations by other workers support the conclusion that both mechanisms were influenced to some extent. Dole has clearly shown a sharp decrease

TABLE V

Metabolic studies three hours after insulin hypoglycemia in cortisone pretreated subjects

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.–F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.–F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs. C. P. 33	Neurosis 1.98	1+	22.2	25.8	3.6	58.5	77.0	18.5	+36.3	+51.2	+14.9
E. K. 29	Neurosis 2.12	1+	12.2	18.2	6.0	23.5	30.8	7.3	+11.3	+12.6	+ 1.3
E. H. 25	Functional G.I. 1.56	1+	67.0	93.0	26.0	122.3	163.3	41.0	+55.3	+70.3	+15.0
W. G. 31	Dermatitis 1.75	1+	49.3	67.0	17.7	94.0	118.2	24.2	+44.7	+51.2	+ 6.5
L. W. 41	Back pain 1.63	1+	52.2	74.0	21.8	93.3	118.3	25.0	+41.1	+44.3	+ 3.2
C. F.	Functional G.I. 1.82	2+	12.5	13.2	0.7	38.0	54.8	16.8	+25.5	+41.6	+16.1
D. M. 34	Neurosis 2.00	1+	11.0	18.7	7.7						
Mean age, 32 Range, 25–41	Mean S.A., 1.84	Mean S.E. S.D.	32.3 8.85 23.0	44.3 12.58 37.70	12.0 3.77 9.8	71.6 15.44 37.83	93.7 7.73 18.94	22.1 4.58 11.22	+35.7 6.31 15.46	+45.2 6.26 15.35	+ 9.50 2.71 6.63
Statistical comparison with control series		p	<0.01	<0.01	<0.01	<0.01	<0.01	>0.5	>0.1 <0.2	>0.5	<0.01
Statistical comparison with hypoglycemic series		p	>0.5	>0.4 <0.5	>0.2 <0.3	>0.5	>0.4 <0.5	>0.1 <0.2	>0.5	>0.5	>0.2 <0.3

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow (ml. blood/min./M.²): Control	During sod. oct.	Δ	Net splanchnic ketone production (μM acetone/min./M.²): Control	During sod. oct.	Δ	Net splanchnic oxygen consumption (ml./min./M.²): Control	During sod. oct.	Δ
yrs. C. P. 33	Neurosis 1.98	1+	1,090	1,164	+ 74	37	206	+169	36	41	+5
E. K. 29	Neurosis 2.12	1+	712	670	− 42	41	49	+ 8	42	33	−9
E. H. 25	Functional G.I. 1.56	1+	1,150	1,098	− 52	299	463	+164	34	43	+9
W. G.	Dermatitis 1.75	1+	814	687	−127	144	170	+ 26	30	25	−5
L. W. 41	Back pain 1.63	1+	1,015	1,071	+ 56	221	268	+ 47	32	40	+8
C. F. 29	Functional G.I. 1.82	2+	1,202	1,019	−183	18	170	+152	40	43	+3

TABLE V—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs. D. M. 34	Neurosis 2.00	1+	1,150			88			40		
Mean age, 32	Mean S.A., 1.84	Mean S.E.	1,019	951	− 46	121.1	221.0	+ 94.3	36.3	37.5	+1.83
		S.D.	71.92	88.41	40.85	40.77	56.32	30.61	1.75	2.66	2.71
Range, 25–41			187.0	216.6	100.1	106.0	138.0	75.0	4.55	6.51	6.63
Statistical comparison with control series		p	<0.01	<0.01	>0.5	<0.01	>0.05 <0.1	>0.1 <0.2	>0.5	>0.3 <0.4	>0.4 <0.5
Statistical comparison with hypoglycemic series		p	>0.02 <0.05	>0.1 <0.2	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones %	Femoral artery glucose mg./100 ml. blood Control	During sod. oct.	Δ	Net splanchnic glucose production mg./min./M.² Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs. C. P. 33	Neurosis 1.98	1+	22	102	92	−10	30	76	+46	80	79	−1
E. K. 29	Neurosis 2.12	1+	1	103	96	− 7	154	61	−93	82	75	−7
E. H. 25	Functional G.I. 1.56	1+	17	78	72	− 6	104	52	−52	71	71	0
W. G. 31	Dermatitis 1.75	1+	3	100	87	−13	95	44	−51	60	59	−1
L. W. 41	Back pain 1.63	1+	5	102	96	− 6	66	58	− 8	75	72	−3
C. F. 29	Functional G.I. 1.82	2+	18	97	95	− 2	36	31	− 5	91	86	−5
D. M. 34	Neurosis 2.00	1+		100			121			60		
Mean age, 32	Mean S.A., 1.84	Mean S.E.	11.0	97.4	89.7	− 5.3	86.6	53.6	−27.2	74.1	73.7	−2.8
		S.D.	3.67	3.38	3.47	2.74	17.88	6.28	19.76	4.45	3.68	1.11
Range, 25–41			9.0	8.79	8.49	6.71	45.2	15.38	48.4	11.57	9.03	2.72
Statistical comparison with control series		p	>0.1 <0.2	<0.01	>0.02 <0.05	>0.2 <0.3	>0.1 <0.2	>0.2 <0.3	>0.1 <0.2	>0.05 <0.1	>0.1 <0.2	>0.5
Statistical comparison with hypoglycemic series		p	>0.5	>0.05 <0.1	>0.1 <0.2	>0.5	>0.5	>0.5	>0.4 <0.5	>0.05 <0.1	>0.05 <0.1	>0.2 <0.3

plasma nonesterified fatty acids (NEFA) after glucose or insulin administration in man (7). This has been confirmed by Gordon and Cherkes (8). More recent observations by Gordon suggest that glucose and insulin decrease the liberation of NEFA from depot fat, leading to a decline in blood NEFA levels and finally to a cessation of extraction by other tissues (9). It is of particular interest that in three of his subjects studied by hepatic catheterization, positive arterial-hepatic venous blood NEFA differences were abolished by glucose administration. On the other hand, the *in vitro* studies of Weinhouse, Millington and Friedman (10) and Lossow, Brown and Chaikoff (11) have demonstrated that glucose prefeeding significantly reduced the conversion of labeled fatty acid substrates to both CO_2 and acetoacetate by liver slices. Furthermore, Lossow and Chaikoff (12) have observed that carbohydrate feeding significantly lowered the conversion of C^{14} of injected fatty acids to CO_2 in intact rats. This effect was far more pronounced in the case of longer-chain fatty acids. However, it should be noted that the longer-chain fatty acids were administered in their experiments as unphysiological, complex emulsions in olive oil, whereas the shorter-chain acids such as octanoate were given as the sodium salt. In similar experiments recently reported by McCalla, Gates and Gordon (13) C^{14} labeled sodium palmitate was administered in the form of a serum albumin-bound complex ion which is believed to be the chemical form of endogenously circulating NEFA. Again, there was a marked inhibition of the conversion of intravenously administered palmitate to CO_2 in the carbohydrate fed intact rat, indicating the occurrence of a block in fatty acid oxidation *per se* at the cellular level. Current evidence suggests that endogenous NEFA are composed almost exclusively of these responsive longer-chain fatty acids (14). Since the carbohydrate sparing of shorter-chain fatty acid (octanoate) oxidation is minimal, we believe that the major carbohydrate effect in our experiments was mediated by a suppression of endogenous fatty acid mobilization or catabolism. In this regard it is noteworthy that the NSKP during the glucose-octanoate infusion experiments was roughly equivalent to the NSKP obtained during the administration of octanoate alone minus the endogenous control period NSKP.

Other data reported herein reveal that hypoglycemia is followed in three to four hours by elevated peripheral venous blood ketone levels, confirming the earlier reports of Collip (15) and Somogyi (16). The basis for this ketonemia in man now seems clearly demonstrated in the hepatic catheterization data in the six subjects which showed a 4.7-fold increase in net splanchnic ketone production three hours after hypoglycemia. This observation lends experimental support to the clinical experience that insulin hypoglycemia may initiate ketosis in the diabetic patient. Since the basal levels of splanchnic ketone production were markedly increased in the posthypoglycemic state, it was at first somewhat surprising to find that octanoate infusion in this group simply induced a net rise in ketone production quantitatively similar to that in the control subjects. This suggests that the ability to utilize the exogenous octanoate for ketone body synthesis is neither augmented nor decreased after hypoglycemia, but rather that the high basal level of ketogenesis is a result either of accelerated utilization of hepatic fatty acids or reflects an increased release of fatty acids from adipose tissue and their delivery to the liver where ketogenesis occurs. Support for the latter concept might be obtained by a study of blood nonesterified fatty acid levels after hypoglycemia, but such data are not recorded in the current literature. The basic biochemical events leading to ketosis in the posthypoglycemic state and their endocrine control have recently been reviewed in detail by one of us (17). The present study throws no light on the endocrine mechanisms involved in the ketonemia consequent to insulin hypoglycemia. However, concurrent studies with rats, to be published elsewhere (18), have shown that this ketosis still occurs in the absence of the pituitary, adrenal cortex and adrenal medulla, and hence hormones from none of these glands are essential. Once ketosis has developed it may be promptly abolished by the administration of a second dose of insulin despite continued hypoglycemia. In fasting hypophysectomized-adrenalectomized rats exhibiting marked hypoglycemia (35 mg. per cent) and ketonemia (258 μM per cent), administration of as little as 0.005 unit of insulin promptly restores blood ketone levels to normal without any further fall in the blood sugar. These data suggest that a relative deficiency in in-

sulin secretion during hypoglycemia may be the single most important hormonal factor contributing to the development of ketosis. It is interesting in this regard that tolbutamide, which reputedly stimulates insulin secretion, is also highly effective in depressing ketonemia during fasting and hypoglycemia (19).

Finally our data show that cortisone pretreatment did not significantly inhibit the elevation in basal net splanchnic ketone production in the posthypoglycemic state. We have pointed out that this interpretation must be accepted cautiously in view of the greater individual variations in this group. However, it is consistent with the recent observation of Amatruda and Engel (18) that cortisone pretreatment does not inhibit the development of ketosis following hypoglycemia in the intact rat. In contrast, it has been shown both in man (20) and the rat (21) that cortisone does inhibit fasting ketosis. Two known metabolic actions of cortisone make it a likely antiketogenic agent in the more slowly developing ketosis of fasting. First, cortisone tends to shift the balance of fat metabolism toward a net accumulation of fat either by inhibiting fat breakdown or stimulating fat synthesis. The second is its action in accelerating carbohydrate production by gluconeogenesis in the liver and in regenerating citric acid cycle precursors from protein catabolism. The answer to the discrepancy in the effect of cortisone on ketosis, suppressing fasting but not modifying posthypoglycemic ketosis, probably resides primarily in the nature of the ketogenic stimulus. It seems likely that the metabolic adjustments rapidly brought forth by the intense stimulus of hypoglycemia outweigh the above-mentioned antiketogenic actions of cortisone, particularly in view of the timing of the present experiments.

An alternate explanation is suggested by the recent finding of Scow, Chernick and Guarco (22) that cortisone is ketogenic in the "totally" pancreatectomized hypophysectomized rat and that this ketosis is prevented by insulin. Cortisone thus may be antiketogenic only when it is capable of bringing about an enhanced rate of insulin secretion. In these terms the variable responses in our cortisone treated posthypoglycemic subjects would reflect variations in the capacity of the pancreas in each case to secrete insulin following suppression by exogenous insulin administration.

SUMMARY

1. Further observations on net splanchnic ketone production (NSKP) were made in metabolically normal human subjects before and after the intravenous infusion of sodium octanoate.

2. The addition of glucose or glucose with insulin to the octanoate infusion significantly lowered the net rise in NSKP noted when octanoate alone was infused.

3. Ketosis in the peripheral blood was observed three hours after the induction of insulin hypoglycemia. While at this time basal NSKP was 4.7-fold greater than in the control subjects, the utilization of infused octanoate was unaffected.

4. Cortisone pretreatment failed to suppress the rise in peripheral blood ketone levels and NSKP occurring in the posthypoglycemic state.

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it may be concluded that a single species of binding site is predominant on transcortin both at 37° C. and at 25° C.

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Regulation of Insulin Secretion by Short Chain Fatty Acids

EVIDENCE is accumulating that in some mammalian species there may be a direct effect of short chain fatty acids on the secretion of insulin from the pancreas. Thus it has been shown that octanoate causes a marked release of insulin from slices of rat pancreas[1]. Furthermore, butyrate was found to evoke a rapid and sustained elevation of serum insulin when infused into adult sheep[2]. These results contrast with the lack of effect on insulin secretion when longer chain fatty acids, such as palmitate, are incubated with pancreas slices[3].

With the development of methods for the isolation of islets of Langerhans in large numbers[4,5] it has become possible to study the mechanism of insulin secretion by the measurement of concentrations of metabolic intermediates. We have measured intracellular concentrations of glucose-6-phosphate following incubation of rat islets with butyrate, octanoate and citrate. The results suggest that short chain fatty acids are important regulators of glucose-6-phosphate levels in pancreatic islet cells, and in this way they may influence insulin secretion.

Islets of Langerhans were isolated from pancreas by collagenase digestion of tissue taken from male albino Wistar rats which had been fasted overnight. The separated islets were divided into groups of ten and incubated in bicarbonate-buffered medium containing albumin together with either glucose, octanoate, butyrate or citrate. In each experiment, the effects of low and high glucose were compared with those of low glucose plus organic acid. After 30 min of incubation at 37° C the medium was sampled for determination of its insulin content by the immunoassay of Hales and Randle[6]. Rat insulin provided standards for the assay. The islets from each group of incubation flasks were then pooled in pointed centrifuge tubes, the medium removed and the islets quickly frozen to −76° C by immersion of the tube in a bath of solid carbon dioxide and ethyl alcohol. They were then subjected to sonication for 20 s, after the addition of perchloric acid (0·1 ml./ten islets). The supernatants from the perchloric extracts were then neutralized with potassium carbonate, before the estimation of glucose-6-phosphate by the enzymatic cycling procedure described by Lowry *et al.*[7]. This gave us reproducible results in good agreement with those already reported by Matchinsky[8] for rat islets. Similar concentrations of glucose-6-phosphate have been reported by Idahl and Hellman[9] for islets taken from obese hyperglycaemic mice, in which β-cells constitute more than 90 per cent of the islet tissue.

The results obtained are shown in Table 1, which shows that butyrate, octanoate and citrate all stimulate the release of insulin from isolated rat islets of Langerhans, and that paralleling the secretion there is an increase in the intracellular concentrations of glucose-6-phosphate in the islets.

These results can be explained as an effect of short chain fatty acids on the intracellular accumulation of citrate which may then affect phosphofructokinase, resulting in an inhibition of glycolysis and accumulation of glucose-6-phosphate, as has been suggested by Randle *et al.* for other tissues[10]. Furthermore, the results do not support the idea that the release of insulin brought about by sugars is mediated by glycolysis, for, in the presence of fatty acids, glycolysis is inhibited, but rather they suggest that glucose has to be metabolized by some other pathway in order to stimulate insulin secretion. We have already indicated that the pentose phosphate pathway may be important in this respect[11].

Table 1. SECRETION OF INSULIN AND THE INTRACELLULAR CONCENTRATIONS OF GLUCOSE-6-PHOSPHATE IN ISOLATED RAT ISLETS OF LANGERHANS INCUBATED IN THE PRESENCE OF OCTANOATE, BUTYRATE AND CITRATE

Addition to medium	Insulin secretion (μ units/10 islets/min; mean ± S.D.)	Glucose-6-phosphate levels (μmoles/10 islets; mean ± S.D.)
2·5 mM glucose	18 ± 4 (20)	25·5 ± 7·0 (8)
20·0 mM glucose	39 ± 6 (20)	59·5 ± 10·0 (8)
2·5 mM glucose + 5·0 mM sodium citrate	37 ± 5 (20)	49·0 ± 12·0 (8)
2·5 mM glucose	18 ± 4 (20)	26·5 ± 5·0 (8)
20·0 mM glucose	38 ± 5 (20)	65·5 ± 11·0 (8)
2·5 mM glucose + 5·0 mM sodium butyrate	33 ± 4 (20)	47·5 ± 10·0 (8)
2·5 mM glucose	19 ± 3 (20)	22·5 ± 5·0 (4)
20·0 mM glucose	40 ± 5 (20)	51·0 ± 5·0 (4)
2·5 mM glucose + 5·0 mM sodium octanoate	38 ± 4 (20)	48·5 ± 7·0 (4)

It is not known whether longer chain fatty acids can also exercise a regulatory effect by this means, because so far it has been difficult to obtain effects of albumin-bound long chain fatty acids on insulin secretion. In addition, ketone bodies have also not been shown to be effective in releasing insulin in most species, whether used *in vitro*[1,12] or *in vivo*[13]. Thus we found that 5 mM β-hydroxybutyrate did not alter rates of secretion when incubated with isolated rat islets. Nevertheless, ketone bodies and long chain fatty acids may be important in modifying insulin release in certain other species, for Madison *et al.* have obtained effects of these agents in inducing insulin secretion in the dog[14,15]. By contrast, a much more consistent effect of short chain fatty acids on release of insulin has been obtained. Our results indicate that the "glucose fatty acid cycle"[10] may be operative as a means of metabolic regulation in pancreatic islet cells.

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Der Ketonkörperstoffwechsel im Hungerzustand und bei chronischer Unterernährung.

Von

Friedrich Müller.

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Mit 7 Textabbildungen.

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Zu den folgenden Untersuchungen wurden wir durch vielseitige Beobachtungen an Dystrophiekranken der Greifswalder Medizinischen Klinik in den ersten Nachkriegsjahren angeregt. Sie sind als die notwendige Ergänzung zu den Gülzowschen Arbeiten über den Kohlenhydratstoffwechsel bei Hunger und Unterernährung gedacht. Die wenigen über den Fettstoffwechsel bei Unterernährung vorliegenden Arbeiten befassen sich weniger mit Fragen des intermediären Stoffwechsels als mit solchen des Fettminimums und der Zufuhr gewisser ungesättigter Fettsäuren (Linol-, Linolen- und Arachidonsäure), die für Wachstum und Wohlbefinden auch des Menschen unerläßlich zu sein scheinen (Lang, Fleisch, Burr u. Burr, Evans u. Lepkovski), oder sie befassen sich mit pathologisch-anatomischen Feststellungen (Fettinfiltration in die atrophisch werdende Leber, Waterlow) und der Registrierung blutchemischer Daten (erniedrigte Werte für Blutfett und Lipoide: Feigl, Overzier, Gülzow). Besonders der niedrige Cholesterinspiegel gilt als Frühsymptom der einsetzenden Inanition (Gülzow).

Wichtiger ist, daß Berning bei dystrophischen Kranken in der Ödemphase beträchtliche Blutketonsteigerungen (im Mittel 11,3 mg-%) fand. Auffallenderweise bestand aber keine Ketonurie, wie sie nach unserer Erfahrung bei solch hohen Werten zu erwarten wäre. Berning hat diese Ketosen in Verbindung zu Gastrektasien und Magenatonien gebracht, wie sie im Verlauf von Dystrophien häufig auftreten können. Er glaubt, daß auch die Komaketosen der Diabetiker und die Narkose- und Hungerketose der frisch Operierten für die dabei so oft vorkommenden Magenatonien verantwortlich zu machen seien.

Demgegenüber haben wir mehrfach darauf hinweisen können, daß die Blutketonwerte von Dystrophikern niedrig liegen. Gülzow fand die Nüchternwerte sogar gelegentlich bis fast auf 0 abgesunken. Auch bei dystrophischen Diabetikern können sich nur im Gefolge deutlicher Stoffwechselentgleisungen höhere Blutketosen entwickeln (Müller). Wodurch sollte es auch zu einem vermehrten Ketonkörperanfall in der Dystrophie kommen? Die Hauptquelle der Ketonkörper, das Fettdepot, ist bis auf geringste, nicht mehr mobilisierbare Reste geschwunden.

Durch die Nahrung wird gleichfalls kaum Fett zugeführt. Und auch der exogene und endogene Eiweißumsatz ist äußerst reduziert.

Berning erklärt seine Dystrophikerketosen durch die gleichzeitige Glykogenverarmung der Leber. Derartige direkte Beziehungen zwischen Keto- und Leberglykogenmangel bestehen aber nicht (vgl. dazu auch Müller: Ketonkörperstoffwechsel). Näher liegt die Deutung, daß sich zur Unterernährung ein akuter Hungerzustand gesellte und so die Einschmelzung des Eiweißbestandes und vielleicht noch vorhandener Fettdepotreste ohne Kohlenhydratschutz erfolgte. Die schweren Enterocolitiden mit Resorptionsstörungen, unter denen so viele Dystrophiker gerade in der Ödemphase zu leiden haben, legen diese Zusammenhänge nahe.

Katsch wies vor einigen Jahren auf die interessante Beobachtung hin, daß bei den unterernährten Diabetikern der Nachkriegszeit solche mit einer konstanten, meist völlig therapieunzugänglichen Restacetonurie vermißt werden. Ebenso ist wohl nur durch den Mangel an ketoplastischen Depots zu erklären, daß das diabetische Koma in diesen Jahren ein verhältnismäßig seltenes Ereignis geblieben ist (Diskussion Katsch-Westphal). Nur in Bezirken katastrophalen Insulinmangels stieg die Komamorbidität und -letalität erschreckend an, aber auch hier nur im Jahre 1946, wie Knick u. Porstmann an Hand des Materials der Leipziger Diabetescentrale nachweisen konnten, so lange nämlich den Diabetikern überhaupt noch einige Fett- und Eiweißreserven für den endogenen Umsatz zur Verfügung standen. Ein Jahr später sank die Komahäufigkeit bei den nunmehr latent oder manifest dystrophischen Diabetikern trotz weiterhin bestehender Insulinverknappung wieder auf das für die letzten Kriegsjahre übliche Niveau ab.

Beim völlig hungernden Menschen liegen die Verhältnisse anders als beim Dystrophiker. Hier kommt es nach Aufbrauch der relativ geringen Kohlenhydratreserven sehr bald zu einer beträchtlichen Ketose, die meist schon mit dem 3. Hungertag ihren Höhepunkt erreicht und dann mit dem Schwinden der Fett- und Eiweißreserven gegen Ende des Hungerzustandes allmählich wieder etwas abzusinken pflegt. Der hungernde Organismus ist hierbei durchaus einem ausschließlich mit Fett und Eiweiß ernährten gleichzusetzen.

Gewisse individuelle Schwankungen im Ausmaß der Ketose bestehen jedoch. So werden offenbar während des Hungerns manche adipöse Menschen, sicher wegen ihrer größeren Depots, leichter und ausgiebiger als magere ketotisch (Folin u. Denis a. a.). Auch unterscheiden sich die verschiedenen Tierarten in Hinsicht auf die Intensität der Ketose während des Hungerns beträchtlich. So bekommen Hunde und Katzen, wie wahrscheinlich alle Fleischfresser, keine Hungeracetonurie (Jost, Minkowski, Baer). Interessanterweise neigt auch das Schwein trotz seiner enormen Fettdepots nicht zur Ketose, was man wohl nur durch eine besondere lipophile Tendenz des Gewebes im v. Bergmannschen Sinne deuten kann.

Funktionelle Untersuchungen über den Fettstoffwechsel fehlen im absoluten Hunger genau so wie bei der chronischen Unterernährung. Wir haben deshalb versucht, mit Hilfe von Stoffwechselbelastungen neue Einblicke in den Ablauf der Fettverbrennung, insbesondere der Ketonkörperbildung und -beseitigung, im Hungerzustand und bei chronischer Unterernährung zu gewinnen.

Leider konnten in den Jahren, in denen uns so reichlich dystrophiekranke Menschen zur Verfügung standen, nur Einzeluntersuchungen durchgeführt werden, da die Beschaffung und insbesondere laufende Ergänzung mancher Chemikalien damals unmöglich war. Auf die viel wichtigeren Verlaufsbeobachtungen an Menschen mußten wir deshalb verzichten und waren gezwungen, mit Hunden zu experimentieren. Dabei war uns sehr wohl bewußt, daß der Hund wegen seiner nur

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geringen Ketoseeignung für Fettstoffwechseluntersuchungen nicht sonderlich geeignet ist, und selbstverständlich deswegen auch Analogieschlüsse zum Fettstoffwechsel des Menschen nur mit Reserve gezogen werden dürfen. Wir wählten trotzdem für unsere Untersuchungen Hunde, da sie als Versuchstiere manche Vorzüge bieten. Wie kaum ein anderes Tier fügt sich der Hund bei richtiger Behandlung fast verständig in den experimentellen Gang der Untersuchungen ein. Abwehr- und Fesselungsreaktionen unterbleiben. Auch haben uns die Ergebnisse der Stoffwechselbelastungen während des absoluten Hungerns sehr bald gezeigt, daß wohl graduelle, jedoch keine prinzipiellen Unterschiede zwischen Mensch und Hund bestehen.

Als Stoffwechseltest wählten wir die i.v. Injektion isotonischer Lösungen von Natriumcapronat bzw. -caprylat. Auf die in mancher Hinsicht physiologischere perorale Fettbelastung verzichteten wir mit Bedacht. Die sehr wechselnden Resorptionsbedingungen der für solche Belastungen immer großen Fettmengen hatten sich uns schon früher bei Untersuchungen an Diabetikern sehr störend bemerkbar gemacht. Um so mehr mußten sie bei der im Verlauf der Versuche sich entwickelnden Sub- und Afermentie hungernder und unterernährter Menschen und Hunde störend ins Gewicht fallen.

Capron- und Caprylsäure eignen sich von den niederen Fettsäuren für i.v. Belastungen besonders gut, da sie kaum oberflächenaktiv auf die Venenwand einwirken, die Injektionen somit immer völlig reizlos ohne Venenschmerz vertragen werden und das ketonämische Maximum später als bei Buttersäure in noch gut faßbarer Zeit nach der Injektion liegt (KRAINICK). Auch Hämolysen, wie sie bei Verwendung der Salze höherer Fettsäuren (gelegentlich schon bei Natriumcaprinatinjektionen) auftreten können, kommen nicht vor.

Für den Menschen wählten wir eine Dosierung von 40 cm³ der isotonischen Lösung, die in 4 min mit gleichbleibender Geschwindigkeit in die Cubitalvene injiziert wurden. Damit gelang es, beim gesunden Menschen regelmäßig eine kurzdauernde geringe Ketose zu erzeugen. Für den Hund von mittlerer Größe genügten schon 20 cm³, die in 2 min in die V. jugularis oder saphena eingespritzt wurden, um jedesmal eine deutliche Ketose zu bekommen. Capillare Blutentnahmen für die Blutketon- und Blutzuckerbestimmungen wurden gewöhnlich 2, 4, 6, 8, 10, 15, 30 und 60 min nach der Injektion der Capronsäure vorgenommen. Die Injektionen haben sämtliche Versuchspersonen gut vertragen. Nur wenige wurden während der Injektion etwas benommen und verspürten einen geringen Hustenreiz mit ranzigem Geschmack im Munde. Bei manchen Hunden setzte eine vertiefte Atmung ein. Sie waren gelegentlich bis zu 10 min nach der Injektion noch torkelig und benommen. Auffallenderweise gewöhnten sie sich aber nach einigen Belastungen an die Säure- bzw. Ketonüberschwemmung. Die anfangs sehr auffälligen Symptome verloren sich allmählich regelmäßig. Die Belastungen wurden jeweils morgens nüchtern 12 Std nach der letzten Mahlzeit bzw. der letzten Fütterung durchgeführt.

Der Ablauf der Capronsäureketose bei gesunden, nicht hungernden Menschen und Hunden.

Vor der Besprechung der Versuchsreihen ist es notwendig, den ketonämischen Normaltyp einer solchen Capronsäure- und Caprylsäurebelastung bei stoffwechselgesunden, nicht hungernden und nicht unterernährten Menschen und Hunden zu betrachten, um für den späteren Vergleich

die physiologischen Varianten des Testes zu kennen. Insgesamt wurden an 12 mischrassigen Hunden mittlerer Größe 33 „Normalbelastungen" gewonnen. Die Normalbelastungen an gesunden Menschen entsprechen in ihren Abläufen den von KRAINICK und später auch von mir veröffentlichten.

Auf einige Beobachtungen, die besonders von KRAINICK und mir bei früheren Untersuchungen zur normalen Capronsäureketose gemacht wurden, soll zunächst kurz hingewiesen werden.

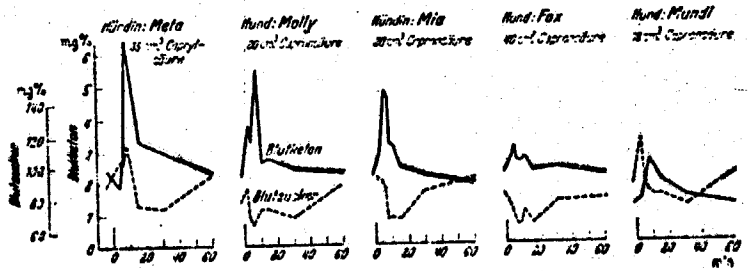

Abb. 1. Caprylsäure- und Capronsäurebelastungen an stoffwechselgesunden, nicht hungernden Hunden.
Aus Raumgründen konnten von den 33 an verschiedensten Hunden gewonnenen Belastungen nur einzelne hier abgebildet werden. Die anderen stehen auf Wunsch zur Einsicht zur Verfügung.

KRAINICK hat 1940 mit $C_{(4)}$—$C_{(10)}$-Fettsäuren, die er i.v. als isotone Natriumseifenlösung injizierte, die β-Oxydation der Fettsäuren auch für den Menschen belegen können. Seitdem wurden ähnliche Fettsäurebelastungen zur Klärung von Fragen des Fett- und Ketonstoffwechsels öfter verwandt (MARKERS und Mitarbeiter, NATH u. BRAHMACHARI). Auch wir haben zu Untersuchungen über die diabetische Ketose und Ketolyse des Menschen und über den Diabetes des Hundes nach Alloxanvergiftung und Pankreatektomie die KRAINICK-sche Fettsäurebelastung gewählt. Die Bildung der Ketonkörper aus den injizierten Fettsäuren im diabetischen Organismus geht praktisch mit der gleichen Geschwindigkeit vor sich wie im gesunden. Das ketonämische Maximum wird 11—12 min nach Beginn der Injektion erreicht, aber die Weiterverbrennung der Ketonkörper, die Ketolyse, ist abhängig vom jeweiligen diabetischen Stoffwechselzustand. Während beim stoffwechselgesunden Menschen und Tier die Blutketonwerte nach Erreichen des Gipfels sehr rasch, spätestens nach 15—30 min wieder zum Ausgangswert absinken, wird beim Diabetiker je nach seinem Stoffwechselzustand ein verzögerter Abfall oder sogar ein weiteres Ansteigen mit Übergang in Hungerketon beobachtet. Zur Illustration des eben Gesagten sind in der Abb. 2 die Capronsäurebelastungen eines Stoffwechselgesunden und eines Diabetikers einander gegenübergestellt.

Gleichzeitig mit den Ketonkörpern steigt bei Diabetikern wie Gesunden in den meisten Fällen auch der Blutzucker an. Es handelt sich dabei um eine immer kurzdauernde hyperglykämische Reaktion auf den Fettsäure- bzw. Ketosereiz infolge einer Glykogenmobilisation, die der reaktiven Hyperglykämie gleichzusetzen ist, die wir zusammen mit KRAINICK schon früher nach peroralen Fettbelastungen beobachteten. Sie ist seitdem auch von anderen Untersuchern beschrieben worden (MARKERS und Mitarbeiter). NATH u. BRAHMACHARI ist es sogar gelungen, durch

wochenlang fortgesetzte Fettsäureinjektionen den Blutzucker dauernd auf erhöhtem Niveau zu halten und schließlich einen Diabetes zu erzeugen.

Die Ketonämie nach Capronsäurebelastung beim gesunden Hund gleicht im wesentlichen der des Menschen. Nur sind die Ausschläge der Ketonkurven wegen der relativ größeren injizierten Fettsäuremengen beim Hunde höher. Die Gipfelwerte unterliegen allerdings von Hund zu Hund beträchtlichen Schwankungen, die sich nicht durch das nur wenig verschiedene Körpergewicht erklären lassen. Die Zusammenstellung einiger typischer, an Hunden gewonnener Normalbelastungen gibt dafür ein gutes Bild (Abb. 1, vgl. besonders Hündin Mia und Hund Fox). Dagegen zeigen die beim gleichen Hunde angestellten Belastungen auch gleichen Kurvenverlauf. Nur gelegentlich differieren die Gipfelwerte gering, da offenbar die Höchstwerte nicht immer erfaßt wurden. Einige Kurvenverläufe zeigen, wie rasch der Anstieg und der Abfall der Ketonwerte erfolgen kann, so daß eine zeitliche Verschiebung der Blutentnahme um nur 30 sec große Höhendifferenzen der Kurve zur Folge haben kann. Die ketonämischen Gipfel werden bei sämtlichen Belastungen innerhalb der ersten 10 min nach beendeter Injektion, bei vielen schon nach 4 und 5 min, erreicht. Meist fallen die Ketonwerte innerhalb von 10, spätestens von 15 min wieder auf den Normalwert ab, der wie beim Menschen zwischen 1,5 und 3 mg-% Gesamtketon liegt. Die Ergebnisse nach Capron- und Caprylsäurebelastung gleichen sich praktisch.

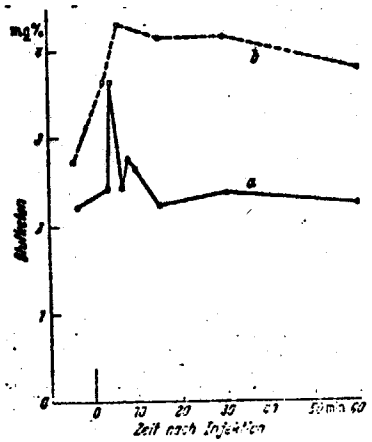

Abb. 2. Capronsäurebelastung bei einem stoffwechselgesunden und einem Diabetiker. a) Ketonkurve des Stoffwechselgesunden (Dr. F.). b) Ketonkurve des Diabetikers (E.F. Kex.-Nr. Harz 8037/47).

Zweifellos spiegelt sich in solchen Kurvenverläufen, *wie aktiv die Fette an den Verbrennungsprozessen auch des nicht hungernden und nicht glykogenverarmten Organismus teilhaben. Sie widerlegen eindeutig die bisherigen Vorstellungen von den „stoffwechselträgen, schwererbrennbaren" Fetten.* Sie zeigen weiter, daß zumindest die exogene Ketonbildung auch beim Hunde ähnlich wie beim Menschen abläuft.

Die Blutzuckerkurven verlaufen weit weniger einheitlich als die Ketonkurven. Zwei Grundtypen herrschen jedoch vor. Der häufigere von beiden zeigt sofort nach der Fettsäureinjektion einen mehr oder weniger ausgeprägten, aber immer kurzdauernden Blutzuckeranstieg, dem ein

Abfall bis unter die Ausgangswerte folgt, gelegentlich bis zu ausgesprochen hypoglykämischen Werten (in einem Fall bis zu 45 mg-%). Bis zum Ende der Belastung nach 60 min hebt sich das Blutzuckerniveau wieder bis zum Ausgangswert oder auch beträchtlich darüber hinaus. Der zweite Typ läßt die primäre hyperglykämische Phase vermissen, die hypoglykämische setzt sofort nach der Injektion ein. Der folgende Wiederanstieg des Blutzuckers gleicht dem des erstbeschriebenen Kurventyps. Außer diesen beiden Formen kommen vereinzelt Varianten vor, die mehr oder minder deutliche Beziehungen zu einer der beiden Formen erkennen lassen.

Der seltenere zweite Kurventyp kommt wohl nur zustande, weil die hyperglykämische Reaktion so rasch abläuft, daß sie gelegentlich durch die Blutentnahme nicht erfaßt wird, oder weil sie durch sehr rasch einsetzende Verbrennungsprozesse ausgeglichen wird. Jedenfalls besteht keine Veranlassung, frühere Feststellungen über den primär hyperglykämischen Effekt durch Fettsäure bzw. Ketonreiz zu revidieren. 24 von den 33 Normalbelastungen am Hund lassen ihn mehr oder minder deutlich erkennen.

Die Deutung der Blutzuckerreaktion nach Fettsäureinjektion ist nicht schwierig. Sie ergibt sich zwanglos aus den bekannten und schon öfter erörterten Korrelationen, die bei der Keton- und Glucoseverbrennung angenommen werden müssen. Auf den Fettsäure- oder Ketonreiz hin wird, dem schnellen Ablauf der Ketogenese entsprechend, durch Glykogenolyse Glucose mobilisiert und zur Verbrennung bereitgestellt (primäre hyperglykämische Phase). Der sofort folgende Blutzuckerabfall bezeugt die aktivierte Verbrennung, durch die auch die Hyperketose rasch wieder zum Verschwinden gebracht wird. Dabei kann es zu einem übermäßigen Absinken des Blutzuckerniveaus im Sinne reaktiver Überleistungen (KATSCH) kommen.

Die Fettsäureverbrennung bei völligem Nahrungsentzug.

Aus den Verläufen der nach Capronsäureinjektionen gewonnenen Blutketon- und Blutzuckerkurven ergaben sich wichtige Aufschlüsse über den Ablauf der Fettsäureverbrennung am stoffwechselgesunden, normalgenährten Organismus. Bei den folgenden Untersuchungen legten wir uns die Frage vor, ob sich während und nach einer Periode völliger Nahrungskarenz die Blutketon- und Blutzuckerkurven der Capronsäurebelastungen ändern und ob gegebenenfalls daraus Rückschlüsse auf einen pathologischen Ablauf der Fettverbrennung gezogen werden können.

Dazu wurden nach genügend langer Vorbeobachtung 3 Hunde einer Hungerperiode von 17—22 Tagen unterworfen, während der sie im Stoffwechselkäfig gehalten wurden und außer Leitungswasser keinerlei Nahrung zugeführt bekamen. Dabei kam es zu Gewichtsverlusten von $^1/_5$ bis $^1/_3$ des Ausgangsgewichtes. In der

sich bei allen 3 Hunden anschließenden Auffütterungsperiode wurde Wert auf besonderen Eiweiß-, Vitamin- und Kalkreichtum der Kost gelegt. Mit Capryl- bzw. Capronsäure wurden die Hunde in Abständen von jeweils wenigen Tagen belastet. Die genauen Daten sind aus den beigefügten Abbildungen zu ersehen. Anfangs glaubten wir, mindestens 30—40 cm³ Fettsäurelösung zu benötigen, um einen ausreichenden ketonämischen Effekt zu erzielen, jedoch sind 20 cm³ völlig ausreichend,

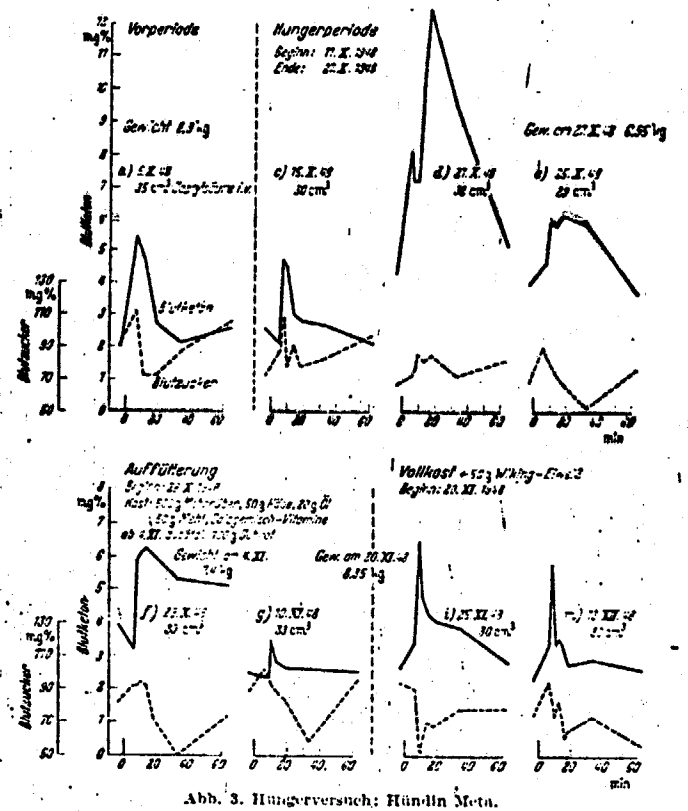

Abb. 3. Hungerversuch: Hündin Meta.

zumal es manchmal bei schlechten oder schon oft benutzten Venen schwierig war, größere Mengen als 20 cm³ mit der erforderlichen Geschwindigkeit von 10 cm³ min einzuspritzen.

Für entsprechende Versuche an Menschen stellten sich freundlicherweise einige Ärzte und Schwestern zur Verfügung. Hierbei mußten wir uns mit wesentlich kürzeren Hungerperioden begnügen, die gewöhnlich am 4. Hungertag endeten. Darnach konnte sofort ad libitum gegessen werden. Die Belastungen wurden jeweils morgens 8 Uhr durchgeführt und mit dem ersten Tag nach der Hungerperiode abgeschlossen. Einige Male wurde doppelt belastet, d. h. nach Ablauf der ersten Belastung wurden

nochmals 40 cm³ Capronsäurelösung injiziert und wie üblich Blutzucker und Blutketon im Capillarblut bestimmt. Weitere Einzelheiten sind aus den Versuchsprotokollen zu ersehen, die wir der besseren Anschaulichkeit wegen zum Teil graphisch aufgezeichnet haben (Abb. 4 und Tabelle 1).

Ergebnisse. Die Abb. 3 zeigt die Caprylsäurebelastungen des Hungerversuches an der Hündin Meta, die 17 Tage hungerte und deren Gewicht in dieser Zeit von 8,9 auf 6,95 kg zurückging. Die Kontrollbelastung der Vorperiode und auch die Belastung 5 Tage nach Beginn der Fastenperiode (Abb. 3a u. c)[1] bieten nichts Besonderes. Die Blutketonverläufe weichen von der Norm nicht ab.

Die Blutzuckerausgangswerte der Hungerbelastungen (Abb. 3c, d, e) liegen etwa 20 mg-% tiefer als die der Kontrollbelastungen. Der Blutzuckerverlaufstyp bleibt aber im wesentlichen erhalten. Nur am Ende der Hungerperiode (Belastung e) sinkt der Blutzucker während der Belastung bis zu dem hypoglykämischen Wert von fast 50 mg-% ab. Mit Beginn der Auffütterung hebt sich das Blutzuckernüchternniveau sofort wieder. Es bleibt aber noch lange eine ausgesprochene Neigung des Blutzuckers bestehen, bis zu Werten von 50 mg-% und darunter abzufallen.

Die Blutketonkurven dagegen verändern sich während des Hungerns deutlich. Schon ihre Ausgangswerte liegen über dem Normalbereich (Abb. 3d, e, f). Bei der am 10. Hungertag durchgeführten Belastung (Abb. 3d) steigt die Ketonkurve doppelt so hoch wie bei den Kontrollbelastungen an. Dabei wird das Maximum nur wenig später als normal erreicht, der Wiederabfall aber verzögert sich deutlich, wodurch die normalerweise schmale und schlanke Ketonzacke auffallend gespreizt wird. Noch 30 min nach Caprylsäureinjektion liegt der Ketonspiegel weit über dem Ausgangswert. Die gleiche Spreizung des Ketonkurvenverlaufs zeigen die letzte Belastung der Hungerperiode (e) und die einen Tag nach begonnener Auffütterung (f). Bei letzterer ist sogar nach 60 min der Ausgangswert noch nicht wieder erreicht. Den hohen Anstieg der Ketonkörper weisen diese Belastungen aber nicht mehr auf. Die übrigen Ketonkurven der Auffütterungsperiode verlaufen bis auf die der Belastungen i und m sehr flach und zeigen einen nur geringen ketotischen Ausschlag. Mit der letzten Belastung (m) dürfte der normale Kurventyp wieder erreicht worden sein.

Der zweite, sich über 22 Tage erstreckende Hungerversuch, bei dem die Hündin Minna über $^1/_3$ ihres Körpergewichtes verlor, und ihre Serumeiweißkörper (Refraktometerwerte) von 5,25 auf 3,5% absanken, läßt an den Ketonkurven prinzipiell die gleichen Veränderungen erkennen, wie sie eben geschildert wurden. Die Morgenketonwerte steigen

[1] Auf die Abbildungen einiger Belastungen dieses Versuches sowie sämtlicher Belastungen der beiden anderen Hungerhunde mußte wegen des zu großen Umfanges verzichtet werden. Sie können zur Einsicht angefordert werden.

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Tabelle 1. *Capronsäurebelastungen bei hungernden Menschen.* (40 cm³ isotonische Na-Capronatlösung i.v., Injektionsdauer 4 min.)

Nr.	Name	Datum	Blutzucker bzw. Blutketon in mg-%								
			nüchtern	2 min	4	6	8	10	15	30	60
						nach beendeter Capronsäureinjektion					
1	Dr. R.	9. 1. 50, 8 Uhr	94	94	91	89	76	80	82	87	98
		Leerversuch	*2,11*	*2,83*	*3,64*	*2,55*	*2,69*	*2,55*	*2,11*	*1,83*	*1,97*
		10. 1. 50, 8 Uhr	64	63	63	59	59	61	66	68	72
		n. d. 1. Hungertag	*6,75*	*7,0*	*7,45*	*8,1*	*8,85*	*9,1*	*8,1*	*7,65*	*7,2*
		11. 1. 50, 8 Uhr	66	63	63	55	66	61	66	66	—
		n. d. 2. Hungertag	*5,4*	*8,35*	*8,85*	*8,35*	*9,35*	*7,2*	*6,55*	*7,45*	*7,0*
		12. 1. 50, 8 Uhr									
		1 Tag nach	84	82	80	85	82	78	87	85	72
		Hungerversuch	*1,97*	*2,38*	*2,69*	*3,0*	*2,55*	*1,97*	*1,97*	*1,42*	*1,69*
2	Dr. Fr.	17. 1. 50, 8 Uhr	91	92	94	96	103	94	98	—	98
		Leerversuch	*2,25*	*2,38*	*3,64*	*2,38*	*2,38*	*2,69*	*2,25*	*2,38*	*2,25*
		19. 1. 50, 8 Uhr	121	93	80	94	82	80	75	75	76
		n. d. 2. Hungertag	*4,65*	*5,8*	*5,95*	*8,1*	*7,85*	*8,85*	*9,35*	*7,65*	*6,35*
		20. 1. 50, 8 Uhr	79	82	86	79	79	73	82	57	77
		n. d. 3. Hungertag	*6,15*	*7,85*	*8,35*	*8,85*	*9,6*	*7,85*	*8,35*	*6,35*	*5,8*
3	G.	7. 2. 50, 8 Uhr	81	92	88	92	81	87	74	87	83
		Leerversuch	*2,55*	*3,3*	*3,16*	*3,47*	*3,6*	*2,83*	*2,69*	*2,38*	*2,25*
		Zweitbelastung		97	96	92	87	81	87	87	87
		im Anschluß an die		*3,0*	*2,83*	*2,83*	*2,55*	*2,69*	*2,55*	*2,25*	*2,38*
		vorhergehende									
		9. 2. 50, 8 Uhr	61	61	65	70	59	70	56	54	61
		n. d. 2. Hungertag	*9,95*	*10,2*	*10,8*	*11,1*	*9,85*	*7,85*	*8,85*	*7,65*	*7,85*
		Zweitbelastung		—	61	61	65	68	66	65	63
		im Anschluß an die		*8,1*	*8,35*	*8,35*	*8,1*	*7,65*	*9,6*	*8,6*	*7,85*
		vorhergehende									
		10. 2. 50, 8 Uhr	74	68	66	68	66	61	72	66	52
		n. d. 3. Hungertag	*10,2*	*10,8*	*11,4*	*11,7*	*12,7*	*11,1*	*10,8*	*10,5*	*10,8*
		8 g Traubenzucker									
		u. 50 g Rohrzucker	150'	210'	270'						
		p. os. i. Anschl. a. d.	120	129	127						
		Belastung	*7,0*	*4,3*	*2,69*						
4	Dr. G.	13. 2. 50, 8 Uhr	95	87	96	—	96	100	98	91	89
		Leerversuch	*2,25*	*2,55*	*2,55*	—	*2,69*	*2,83*	*2,69*	*2,38*	*2,11*
		Zweitbelastung		104	102	93	105	104	102	84	87
		i. Anschluß an die		*2,25*	*2,55*	*2,69*	*2,38*	*2,25*	*2,69*	*2,11*	*2,11*
		vorhergehende									
		14. 2. 50, 8 Uhr	96	92	—	103	96	97	83	97	94
		n. d. 1. Hungertag	*2,55*	*3,0*	*3,47*	*3,64*	*3,47*	*3,3*	*2,55*	*2,38*	*2,25*
		Zweitbelastung		96	76	103	99	88	92	92	90
		i. Anschluß an die		*2,83*	*3,0*	*3,16*	*3,3*	*2,69*	*2,83*	*2,38*	*2,11*
		vorhergehende									
		15. 2. 50, 8 Uhr	91	76	87	83	87	87	85	78	78
		n. d. 2. Hungertag	*4,65*	*5,95*	*7,2*	*7,9*	*8,1*	*6,55*	*5,95*	*5,55*	*5,2*
		16. 2. 50, 8 Uhr	65	78	74	81	72	74	78	72	72
		n. d. 3. Hungertag	*11,1*	*13,0*	*13,7*	*13,0*	*14,2*	*11,5*	*13,0*	*11,7*	*11,1*
		Zweitbelastung		69	74	71	67	67	71	72	74
		i. Anschluß an die		*14,1*	*11,9*	*16,7*	*13,7*	*14,5*	*13,0*	*11,4*	*10,2*
		vorhergehende									
		17. 2. 50, 8 Uhr									
		1. Tag nach	133	138	142	—	125	146	151	129	112
		Hungerversuch	*1,97*	*2,25*	*2,55*	—	*2,69*	*2,83*	*2,38*	*2,55*	*2,11*
		Zweitbelastung									
		i. Anschluß an die		110	107	105	107	98	103	105	100
		vorhergehende		*2,38*	*2,55*	*2,69*	*2,25*	*2,11*	*2,11*	*1,83*	*1,97*

mit Beginn der Hungerperiode an und erreichen etwa am 10. Hungertag ein Niveau von 4.5—5.5 mg-%, das dann bis zum Ende des Hungerzustandes beibehalten wird. Bis auf eine zeigen sämtliche Belastungen während des Hungerzustandes die beschriebene typische Spreizung der Ketonzacke, die auch hier allein durch den verzögerten Abfall der Ketonwerte entsteht; denn das ketonämische Maximum wird immer ebensoschnell (spätestens 10 min nach beendeter Capronsäureinjektion) wie bei den Kontrollbelastungen der Vorperiode erreicht. Auch hier überragt

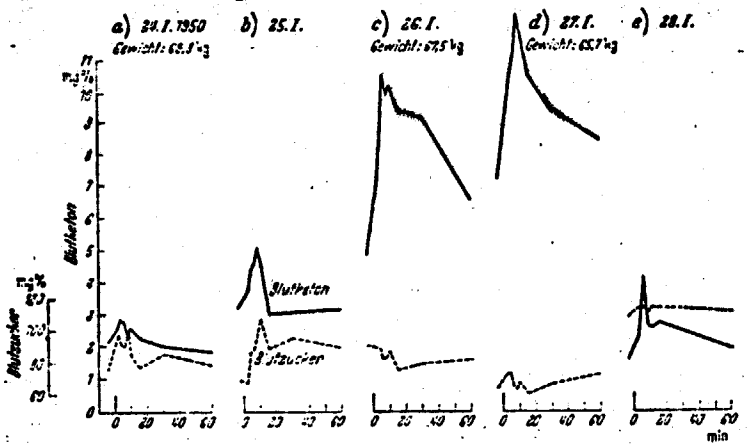

Abb. 4. Hungerversuch: Schwester K. B. Hungerperiode vom 21. 1. 50 bis zum Ende der Belastung am 27. 1. 50.

der ketonämische Gipfel einer Hungerbelastung die übrigen fast um das Doppelte. Bereits einen Tag nach Beginn der Wiederauffütterung beginnt die Ketonkurve mit einem völlig normalen Nüchternketonwert, fällt allerdings von schnell erreichter Höhe noch deutlich verzögert ab. Die anderen Belastungen der Nachperiode zeigen nichts Besonderes.

Die Blutzuckerkurven verlaufen nicht sehr charakteristisch. Immerhin sind die beim ersten Hungerversuch beschriebenen Veränderungen wenigstens angedeutet vorhanden, so die allgemeine Blutzuckerdepression und die Neigung zum Absinken auf hypoglykämische Werte während der Belastungen.

Die Belastungen des dritten Hungerversuches zeigen die in der Hungerphase auftretenden Veränderungen nicht so typisch. Sie sind nur angedeutet, so der verzögerte Abfall der Ketonwerte bei einigen Belastungen und der alle anderen deutlich überragende Ketongipfel am 13. Hungertag.

Von den 5 an hungernden Menschen durchgeführten Versuchsreihen wählten wir zur Abbildung nur eine aus (Abb. 4), die übrigen, deren

Ergebnisse den beiden abgebildeten weitgehend entsprechen, sind in der Tabelle 1 protokolliert.

Die Morgenketonwerte steigen während des Hungerns bis zum 4. Tage an. Der Blutzucker fällt dagegen regelmäßig etwas ab. Auch die Belastungsketosen steigern sich mit fortschreitendem Hunger beträchtlich. Dabei werden die Ketongipfel wieder mit derselben Geschwindigkeit (bei den abgebildeten Versuchen spätestens 8 min nach beendeter Injektion), ja gelegentlich sogar früher als bei den Kontrollbelastungen erreicht. Der Wiederabfall der Ketonkörper verzögert sich jedoch zunehmend, und die daraus resultierende Spreizung der Ketonzacke prägt sich stärker aus. Bei einigen Belastungen liegen selbst die Endwerte nach 60 min noch weit über den Ausgangswerten (Abb. 4c u. d). Die abschließenden Belastungen, einen Tag nach dem Ende der Hungerperiode, zeigen gewöhnlich sofort wieder die gleichen Ketonkurven wie vor Beginn des Hungerns. Nur selten ist der ketonämische Ausschlag der Kurve noch etwas höher als normal (Abb. 4e).

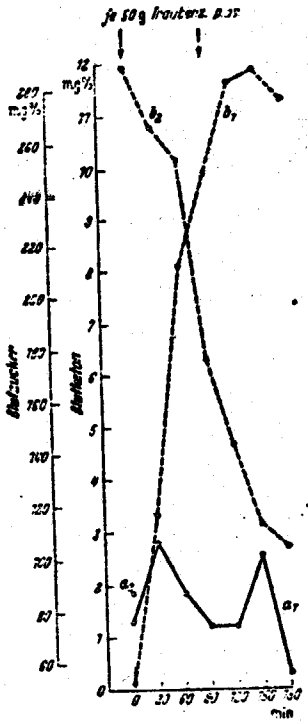

Abb. 5. Traugott-Staub Traubenzuckerdoppelbelastung nach einer Hungerperiode von 2 Tagen (Dr. R.). a_2: Blutzuckerkurve der Kontrollbelastung. a_1: Nüchternketonwert am Tage der Kontrollbelastung. b_1: Blutzuckerkurve der Bel. nach Hungerperiode. b_2: Blutketonkurve der Bel. nach Hungerperiode.

Auffallend hoch, wesentlich über dem üblichen Nüchterniveau, kann manchmal der Blutzucker dieser Schlußbelastungen liegen. So beginnt die Blutzuckerkurve einer solchen (Tabelle 1, Nr. 4) bei 132 mg-% und steigt bis zu 155 mg-% an. Sie fällt allerdings im weiteren Verlauf des Versuches bis auf normale Werte ab und reagiert auch auf die sich anschließende Zweitbelastung nicht wieder mit einem Anstieg. In einigen Fällen stellten wir am Ende des Hungerzustandes Traugott-Staubsche Glucosedoppelbelastungen an. Die Blutzuckerkurven solcher Belastungen verlaufen dann wie bei einem Diabetes mellitus. Sie zeigen einen sehr hohen Blutzuckeranstieg und einen noch höheren Blutzuckerzweitgipfel. Der Blutketonspiegel sinkt während der Belastung stetig ab und erreicht bald sein normales Niveau. Ein besonders schönes Beispiel bringt die Abb. 5.

Besprechung der Ergebnisse. Im folgenden wollen wir versuchen, den Ursachen der im Hungerzustand von der Norm abweichenden Veränderungen des Ketonstoffwechsels nachzugehen. Bei einem solchen Deutungsversuch läßt sich eine gewisse Spekulation nicht vermeiden. Zu viele, oft nicht zu trennende Stoffwechselvorgänge interferieren: Geschwindigkeit und Ausmaß der Verbrennungsprozesse, vermehrte Ketogenese und verminderte Ketolyse oder umgekehrt, Ablagerung der injizierten Capronsäure in den Fettdepots oder zusätzliche Mobilisation von Depotfett durch Reiz u. a.

Während des Hungerzustandes fallen drei typische, bei Hund und Mensch fast in gleicher Weise auftretenden Merkmale auf:

1. eine allgemeine *Zunahme der Blutketonkörper*,
2. eine *Verzögerung im Wiederabfall der Ketonwerte nach der Capronsäurebelastung*, schließlich
3. eine *beträchtliche Steigerung des ketonämischen Belastungseffektes* beim Menschen, zu der es beim Hunde nur in gewissen Phasen der Hungerperiode kommt.

Die allmähliche Zunahme der Blutketonkörper im Hunger bis zu einem erhöhten und dann bis zum Ende des Hungerzustandes meist gleichbleibenden Niveau ist seit langem bekannt. Von gewisser Bedeutung ist aber, daß sich auch bei manchen Hunden der Blutketonspiegel etwas hebt. Die Ausscheidung von Ketonkörpern durch die Nieren steigt dabei aber nicht so hoch an, daß die LEGALsche Probe positiv wird. Trotz dieser zweifellos nur geringen Ketose sinkt der RQ soweit ab, daß auch hier sicher nur Fett und Eiweiß verbrannt werden können (SNAPPER u. v. CREFELD). Daraus muß aber der Schluß gezogen werden, daß sich während des Hungerns beim Hunde Ketogenese und Ketolyse besser die Waage halten als beim Menschen und so eine Ketonstauung vermieden wird. Das kann daran liegen, daß sich der allgemeine Stoffwechselumsatz des Hundes während des Hungerns ausgiebiger und schneller als beim Menschen auf ein reduziertes Sparniveau einreguliert und sich etwa dem eines winterschlafenden Tieres nähert, oder daran, daß der Hund nur geringe Fettdepots zur Verfügung hat und deshalb sehr bald auf seinen Eiweißbestand als Energielieferanten angewiesen ist, bei dessen Abbau aber bekanntlich außer den Ketonkörpern auch reichlich Glucose als „antiketogene" Substanz anfällt. Die theoretische dritte Möglichkeit, daß der Hund ganz allgemein eine größere Fähigkeit zur Ketolyse besäße als der Mensch, findet an unseren Untersuchungen keine Stütze, da die an nicht hungernden Hunden gewonnenen Capronsäureketosen bei Berücksichtigung der verschiedenen Körpergewichte und Capronsäuremengen im Vergleich zu den an Menschen gewonnenen durchaus äquivalente Höhen erreichten.

Die für den Hungerzustand so charakteristische Abwandlung des Ketonkurventyps nach Capronsäurebelastung, der hohe Anstieg und das träge verzögerte Absinken der Ketose, läßt sich am zwanglosesten durch eine Hemmung der Ketolyse deuten. Die Ketogenese in der Leber ist dagegen offenbar nicht gestört, da selbst die hohen Ketonmaxima in der gleichen kurzen Zeit nach der Injektion wie bei Nichthungernden erreicht werden.

Die Hungerketonkurven stimmen mit denen, die wir früher an Diabetikern und diabetischen Hunden gewannen, völlig überein (Abb. 6), so daß sich die Frage aufdrängt, ob den gleichen Ketonkurventypen auch gleiche Störungen im Stoffwechsel entsprechen.

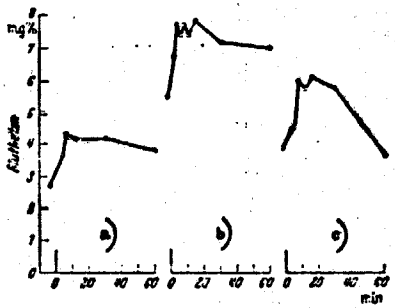

Abb. 6. Gegenüberstellung von Capronsäurebelastungen bei Diabetes mell. und im Hungerzustand. a) Ketonverlauf nach Belastung bei einer stoffwechselmäßig gut eingestellten Diabetikerin (EF Bez. Nr. Garz 863/47). b) Belastung nach Pankreatektomie. Hündin Mia. c) Belastung nach 13tägig. Hungerzustand. Hündin Meta.

Beiden Stoffwechselzuständen gemeinsam ist die Zuckernot der Gewebe; beim Diabetes infolge relativen oder absoluten Insulinmangels bei hohen Blutzuckerwerten, beim Hunger infolge effektiven Kohlenhydratmangels nach raschem Aufbrauch der geringen Reserven und offenbar nur zögernder Neubildung, wie man aus dem Absinken des Blutzuckerniveaus während des Hungerns auf fast hypoglykämische Werte schließen muß. Deshalb beseitigt auch schon geringe Zuckerzufuhr selbst hohe Hungerketosen sofort (Tabelle 1, Nr. 3).

Neben dem Kohlenhydratmangel kommen für die Ketolyschemmung im Hunger noch zwei weitere Faktoren in Betracht: *Ein allgemeiner Zell- und Gewebsschaden*, der mit fortschreitendem Hunger immer stärker zur Geltung kommen dürfte, *und ein relatives Insulindefizit* infolge allgemeiner Stoffwechseleinsparung und bei länger anhaltendem Hunger auch infolge Inaktivität und Hungeratrophie der Langerhans-Inseln. Außerdem gehört Insulin zu den Proteohormonen, zu deren Bildung hochwertige Eiweißkörper benötigt werden, die bei längerem Hunger fehlen. Insulinmangel und Gewebsschaden wirken etwa im gleichen Sinne hemmend auf den Stoffwechsel. Mit unserer Versuchsanordnung lassen sie sich anteilmäßig nicht trennen. Ihre wahrscheinliche Mitbeteiligung an den beschriebenen Hungerstoffwechselveränderungen ergibt sich aber aus folgenden Beobachtungen: 1. bleibt bei einigen Versuchen auch am Tage nach begonnener Wiederauffütterung der pathologische Ablauf der ketonämischen Belastungskurven zumindest angedeutet erhalten,

obwohl sich offenbar das Kohlenhydratdefizit ausgeglichen hat (normale oder erhöhte Blutzuckerlage!) (unter anderen Abb. 3f, 4e); 2. liegt auch der Blutzucker an diesem Belastungstag manchmal auffallend hoch und steigt zudem unter der Capronsäurebelastung noch weiter beträchtlich an (Abb. 4e, Tabelle 1, Nr. 4); 3. verlaufen Traugott-Staubsche Glucosebelastungen am Ende einer Hungerperiode ausgesprochen diabetisch (Abb. 5). Die beiden letzten Beobachtungen entsprechen ähnlichen GÜLZOWS und sind analog den Hungerglykosurien HOFMEISTERS und den Vaganten-Glykosurien HOPPE-SEYLERS zu werten.

Die Fettverbrennung bei chronischer Unterernährung.

Der Stoffwechsel des absolut hungernden Organismus erhält, wie wir gesehen haben, seine charakteristische Note durch den schnell einsetzenden Mangel an Kohlenhydraten. Auch die Neubildung von Zucker aus Eiweiß und vielleicht auch aus Fett bleibt ganz offenbar hinter dem Bedarf zurück. So wird der Hungerstoffwechsel im wesentlichen von den vorhandenen Fett- und Eiweißdepots bestritten. Bei der chronischen Unterernährung mit allmählich einsetzender Dystrophie liegen dagegen die Verhältnisse anders. Kohlenhydrate stehen hier trotz der Unterernährung immer reichlich zur Verfügung, häufig sogar reichlicher als bei normaler Kost, wenn auch die notwendige kalorische Gesamtenergie im allgemeinen durch sie nicht garantiert wird. Fett und Eiweiß werden aber mit der Nahrung kaum zugeführt, so daß es auch hier, wie bei absolutem Hunger, zu einer, allerdings langsameren Einschmelzung der Fett- und Eiweißbestände kommen muß, deren Verbrennung aber unter dem Schutz einer genügenden Kohlenhydratverbrennung vonstatten geht.

Für die vorliegenden Untersuchungen scheint uns deshalb die Beantwortung der Frage von besonderem Interesse, ob der Fettstoffwechsel auch bei chronischer Unterernährung trotz genügender Kohlenhydratzufuhr ähnlich von der Norm abweicht wie bei absolutem Hunger.

Ergebnisse. Leider war es nicht möglich, Verlaufsbeobachtungen an dystrophischen Menschen durchzuführen. Die Ergebnisse der während der Auffütterung an Dystrophikern gewonnenen Einzeluntersuchungen sind aus der Tabelle 2 zu ersehen. Sie bestätigen Bekanntes. Auch hier liegt das Nüchternketon der Dystrophiker niemals über, sondern häufig sogar unter der Norm. Ebenso lassen Adrenalin- und Capronsäurebelastungen keinerlei besondere Ketoseneigung erkennen (Tabelle 3 u. 4). Nur bei einer Belastung (Tabelle 3, Nr. 6) wird der normale Ketonbereich eben überschritten. Auch bei den beiden lipophilen Dystrophikerinnen (Tabelle 3, Nr. 4 u. 5) kommt es nach Adrenalininjektion nicht zur Ketose. Es ist daher möglich, daß die besondere lipophile Neigung dieser dystrophischen Fettsucht-formen zu gewissen Phasen jegliche zusätzliche

Tabelle 2. *Nüchternketonwerte von Dystrophikern.*

Nr.	Name	Datum	Gewicht kg / Größe m	Serumeiweiß %	Blutketon mg-%	Blutzucker mg-%	Bemerkungen
1	Anton K. Reg.-Nr. 1739/47	22. 11. 47	59,8 1,76	6,96	*1,83*	69	Wiederauffütterungsphase
2	Helmut D. Reg.-Nr. 2721/47	27. 11. 47	56,9 1,71	4,94	*1,14*	76	Nach Ödemausschwemmung
3	Otto H. Reg.-Nr. 2293/47	28. 11. 47	44,2 1,69	6,87	*2,38*	82	Durchfallphase mit Bluteindickung
4	Charlotte B. Reg.-Nr. 3101/47	6. 1. 48	50,8 1,80	4,94	*1,83*	96	Ödemphase
5	Kurt B. Reg.-Nr. 3446/47	13. 2. 48 17. 2. 48 21. 3. 48 6. 4. 48 13. 4. 48	52,5 1,68	4,94	*1,55* *1,38* *1,12* *1,55* *1,12*	70 73 63	Präinaniertes u. Inanition
6	Traute K. Reg.-Nr. 337/48	5. 4. 48 9. 4. 48	68,0 1,60	8,24	*1,12* *1,55*	88 93	Lipophile Dystrophie
7	Erna D. Reg.-Nr. 2748/48	2. 6. 48	75,0 1,56	6,86	*1,55*	74	Lipophile Dystrophie
8	Max Sch. Reg.-Nr. 3040/50	23. 11. 50	40,2 1,73	4,87	*2,55*	69	Nach Ödemausschwemmung
9	Bruno D. Reg.-Nr. 1166/49	1. 12. 49	55,7 1,65	5,83	*1,55*	72	Feuchte Dystrophie

Fettverbrennung verhindert, während ja sonst Fettsüchtige eher eine verstärkte Adrenalinketose aufweisen (MÜLLER).

Für die Untersuchungen an dystrophischen Hunden wählten wir drei mischrassige Tiere mittleren Gewichtes aus. 2 Hunde wurden nach Ausprägung schwersten dystrophischen Zustandes (nach 52 bzw. 38 Tagen

Tabelle 3. *Adrenalinbelastung bei Dystrophikern* (1 mg Adrenalin subcutan). (Die Daten für Gewicht, Größe und Serumeiweißkörper sind aus Tabelle 2 zu ersehen.)

Nr.	Name	Datum	Blutzucker bzw. *Blutketon* in mg-% nüchtern	15 min	30	60	90	120	150	180
			nach Adrenalininjektion							
1	Anton K.	22. 11. 47	69 *1,83*	60 *1,11*	100 *1,07*	100 *1,99*	86 *1,11*	82 *1,83*	78 *1,38*	94 *1,11*
2	Otto H.	28. 11. 47	82 *2,38*	86 *2,69*	100 *2,83*	135 *2,93*	128 *2,55*	89 *2,25*	89 *2,25*	71 *2,11*
3	Kurt B.	17. 2. 48	73 *1,28*	68 *1,69*	82 *1,55*	111 *1,42*	110 *1,42*	108 *1,55*	92 *1,69*	83 *1,42*
4	Traute K.	3. 4. 48	88 *1,42*	107 *1,42*	107 *1,55*	111 *1,42*	85 *1,42*	116 *1,55*	81 *1,28*	125 *1,42*
5	Erna D.	2. 6. 48	74 *1,55*	88 *2,11*	84 *2,83*	77 *2,25*	68 *1,55*	83 —	52 *2,11*	77 *2,69*
6	Max Sch.	23. 11. 50	69 *2,55*	74 *2,83*	76 *2,83*	83 *3,00*	81 *3,3*	78 *3,16*	76 *2,83*	67 *2,55*

Tabelle 4. *Capronsäurebelastungen bei Dystrophikern* (50 cm³ Capronsäurelösung i.v. Injektionsdauer 4 min).

Nr.	Name	Datum	Blutzucker bzw. *Blutketon* in mg-% nücht.	3 min	7	15	30	60	90
			nach Capronsäureinjektion						
1	Charlotte B.	6. 1. 48	96 *1,83*	105 *1,55*	103 *1,55*	103 *1,83*	62 *2,11*	82 *1,83*	96 *1,83*
2	Helmut D.	27. 11. 47	76 *1,14*	71 *1,38*	69 *1,42*	80 *1,69*	94 *1,38*	94 *1,38*	91 *1,69*

Unterernährung) durch Ausbluten getötet. Beide zeigten bei der Sektion eine ausgesprochene Fettcirrhose der Leber. Bei dem 3. Hund wurde eine Auffütterungsperiode angeschlossen, während der wir die Capronsäurebelastungen fortsetzten. Die Belastungen selbst wurden regelmäßig nüchtern 8 Uhr morgens durchgeführt. Einzelheiten der Versuchsanordnung, der Kost, der Daten über Gewicht usw. sind aus dem abgebildeten Protokoll (Abb. 7) ersichtlich[1].

[1] Aus Raumgründen konnten von den 23 während des Versuches angestellten Belastungen nur einzelne typische in der Abbildung dargestellt werden. Auch auf die graphische Wiedergabe der beiden anderen Dystrophieversuche mußte aus dem gleichen Grunde verzichtet werden. Die abgebildeten Protokolle stehen Interessenten zur Einsicht zur Verfügung.

Hund Molly (Abb. 7): An eine etwa einen Monat währende Vorperiode mit gewöhnlicher, ausreichend Eiweiß enthaltender Vollkost, an deren Ende der Hund 6,5 kg wog und einen Serumeiweißspiegel von 5,60% aufwies, schloß sich eine Unterernährungsperiode von 98 Tagen mit einer ausgesprochenen Mangelkost, bestehend aus 250 g Wruken, 25 g Mehl und 5 g Fett (20 g Kohlenhydrate, 6 g Eiweiß, 6,0 g Fett, 159 Cal.) an, in der das Gewicht des Hundes zunächst relativ schnell, später deutlich langsamer und z. T. ausgeglichen durch Wassereinlagerung bis auf 4,3 kg abnahm. Die Serumeiweißkörper sanken zunächst bis auf 3,3% ab, nahmen aber bis zum Ende der Dystrophieperiode, sicher infolge Bluteindickung, wieder bis

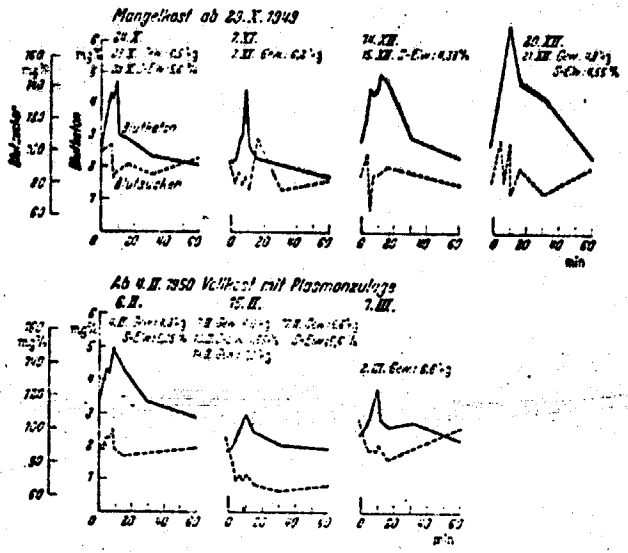

Abb. 7. Unterernährungsversuch Hund Molly. Ab 29. 10. 49 Mangelkost: 250 g Wruken, 25 g Mehl, 5 g Fett (20 g Kohlenhydrate, 6 g Eiweiß, 6 g Fett, insgesamt 159 Kalorien) Ab 4. 2. 50 Vollkost mit 50 g Plasmonzulage. Zu den Belastungen jeweils 20 ccm Isot. Na-Capronatlösung i.v.

zu 5,08% zu. Während der Auffütterung (Vollkost + Eiweißzulage von 50 g Plasmon) nahm der Hund rasch wieder an Gewicht zu und lagerte reichlich Fett ab. Er verblieb in diesem lipophilen Stadium, welches als überkompensierendes Regulationsgeschehen der lipophilen Dystrophie des Menschen völlig gleichzusetzen ist, bis zum Ende des Versuches nach etwa einem Monat. Auch das Gewicht des Hundes lag in dieser Phase bis Versuchsende höher als ausgangs.

Die Blutzuckerkurven verliefen während Unterernährung und Wiederauffütterung unauffällig. Nur bei 2 Belastungen zu Beginn der Unterernährungsperiode traten hypoglykämische Werte von fast 50 mg-% auf.

Die Ketonkurven dagegen zeigen dieselben Veränderungen, allerdings nicht so regelmäßig und gelegentlich nur angedeutet, wie wir sie bei den völlig hungernden Hunden beschrieben haben. Besonders bei unserem als

Beispiel ausgewählten Hund kommt mit fortschreitender Dystrophie die Verzögerung im Wiederabfall der Capronsäureketose und die daraus resultierende Spreizung der Ketonkurven deutlich zur Geltung. Nach Beginn der Wiederauffütterung gleicht sich die Spreizung des Ketonkurvenverlaufes sofort wieder aus. Ebenso wie bei den Hungerhunden fallen auch hier einzelne Belastungen mit besonders hohen Ketongipfelwerten auf, wie sie weder in den Vor- und Nachperioden, noch bei Belastungen an anderen nicht hungernden Hunden beobachtet wurden (vgl. Abb. 1). Bei fast sämtlichen Belastungen der Auffütterungsperiode dagegen, besonders während der lipophilen Ansatzphase dieses Hundes liegen die Ketongipfel auffallend niedrig.

Besprechung. Bei dystrophischen Hunden können ähnliche Ketolysehemmungen im Ablauf der Capronsäureketosen auftreten wie im absoluten Hunger und beim Diabetes mellitus. Ein grob rechnerischer Überschlag — unter Zugrundelegung der Shafferschen Fett- und Kohlenhydratrelation — ergibt sofort, daß genügend Kohlenhydrate mit der Nahrung zugeführt wurden, um die vollständige Verbrennung des einschmelzenden Depotfettes und Körpereiweißes zu garantieren. Der kalorische Index lag am Ende der Dystrophieperiode des Hundes Molly bei 37. Auch das Absinken des Blutzuckerniveaus konnte vermieden werden. *Ich halte deshalb den Schluß für erlaubt, daß die Hemmung der Ketolyse bei chronischer Unterernährung mit Dystrophie nicht durch Mangel an Kohlenhydraten wie bei absolutem Hunger, sondern vorwiegend durch ein Absinken der Insulinproduktion und durch Verschlechterung des Stoffwechselumsatzes infolge allgemeinen Zellschadens verursacht wird.* Die Frage, welchem von den beiden Faktoren, Insulinmangel oder Gewebsschädigung, dabei eine größere Bedeutung zukommt, bleibt solange müßig, bis es gelingt, auch kleine Mengen von aktivem Insulin im Blut nachzuweisen. Dystrophische Menschen bekommen, wie wir oben schon sahen, nur deshalb keine Ketose, weil ihnen weder in der Nahrung noch in den Depots nennenswerte Mengen ketoplastischer Grundstoffe zur Verbrennung bereitstehen. *Die Ketolysehemmung in der Dystrophie kann erst nach Fettbelastung evident werden.*

Die geringen ketotischen Ausschläge der Capronsäurebelastungen in der Auffütterungsperiode finden ziemlich zwanglos durch die gleichzeitig vorhandene lipophile Tendenz des Fettgewebes ihre Deutung. Offenbar wird die injizierte Capronsäure gar nicht nennenswert verbrannt, sondern sofort in den Depots abgelagert. Ebenso muß das Ausbleiben jeglicher ketotischer Belastungsreaktion während der Auffütterung bei dystrophischen Menschen (Tabelle 3 u. 4) aufgefaßt werden.

Es sei nochmals auf die häufigen Parallelen hingewiesen, die sich bei Hunger und Unterernährung zwischen dem Stoffwechsel von Kohlenhydraten und von Fetten ergeben. *Der Stoffwechsel beider Stoffe zeigt bei absolutem Hunger und in der Dystrophie Veränderungen, die denen beim Diabetes mellitus weitgehend gleichen.* Nur das Verhältnis der jeweils auslösenden Faktoren ändert sich. Bei Diabetes stehen Insulinmangel und daraus sich ergebende Zuckernot der Gewebe im Vordergrund, ein sicher auch vorhandener Gewebsschaden tritt im allgemeinen zurück und kommt erst in der Stoffwechseldekompensation, im diabetischen Koma, zur Geltung (MÜLLER). Anders bei absolutem Hunger. Hier spielt fraglos der effektive Kohlenhydratmangel die Hauptrolle, und Insulindefizit und Gewebsschädigung sind demgegenüber belangloser. In der Dystrophie sind es jedoch gerade diese beiden Faktoren, die an der Fettverbrennungsstörung teilhaben.

Für den Kohlenhydratstoffwechsel sind die aufgezeigten Beziehungen zwischen Hunger, Unterernährung und Diabetes schon vor Jahrzehnten von HOFMEISTER (Hungerglykosurie bei unterernährten Hunden) und von HOPPE-SEYLER (Vagantenglykosurie) vermutet worden. In letzter Zeit ist besonders GÜLZOW dieser Frage in mehreren Arbeiten nachgegangen und hat die vermuteten Beziehungen gesichert. *Wir konnten zeigen, daß nicht nur die Kohlenhydrate, sondern auch die Fette, also der gesamte Energiestoffwechsel in gleicher Weise beim Diabetes mellitus, im absoluten Hunger und bei chronischer Unterernährung reagieren.*

Zusammenfassung.

Die Stoffwechselvorgänge im Hunger und in der Unterernährung wurden bisher im wesentlichen nur von den Kohlenhydraten und den Eiweißen her betrachtet. Wir haben versucht, mit Hilfe von Stoffwechselbelastungen neue Einblicke in den Ablauf auch der Fettverbrennung, der Ketogenese und Ketolyse, im akuten Hungerzustand und bei chronischer Unterernährung zu gewinnen.

Vorversuche mit Hilfe von Capronsäurebelastungen an stoffwechselgesunden, nicht hungernden Hunden und Menschen zeigten erneut, wie aktiv und rasch die Fette an den Verbrennungsprozessen auch des nicht hungernden und nicht glykogenverarmten Organismus teilhaben. Sie widerlegten eindeutig die bisherigen Vorstellungen von den „stoffwechselträgen und schwerverbrennbaren" Fetten.

Capronsäurebelastungen an akut hungernden Hunden und Menschen ergaben eine auffallende Verzögerung im Wiederabfall der Ketonwerte nach der Belastung, die sich am zwanglosesten nur durch eine Hemmung der Ketolyse deuten ließen. Außerdem zeigte sich besonders beim Menschen eine beträchtliche Steigerung des ketonämischen Effektes.

Auch an dystrophischen Hunden wurde die gleiche Ketolyschemmung im Ablauf der Capronsäureketose beobachtet, obwohl genügend Kohlenhydrate mit der Nahrung zugeführt wurden. Es wird deshalb der Schluß gezogen, daß die Hemmung der Ketolyse bei chronischer Unterernährung

mit Dystrophie nicht durch Mangel an Kohlenhydraten wie beim absoluten Hunger, sondern vorwiegend durch das Absinken der Insulinproduktion und durch Verschlechterung des Stoffwechselumsatzes infolge allgemeinen Zellschadens verursacht wird.

Sowohl bei absolutem Hunger als auch bei der Dystrophie haben sich damit Veränderungen im Fettstoffwechsel ergeben, die denen beim Diabetes mellitus weitgehend gleichen.

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Dozent Dr. FRIEDRICH MÜLLER, Medizinische Universitätsklinik, Greifswald.

Aus dem Physiologischen Institut d
(Direktor: Prof. Dr. med. u. I

Beitrag zur Frage der Bl nach intravenöser Dex

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Seitdem das tiefere Verständnis un gänge auf der Lehre von den Rhythm dem Schwerpunktswechsel der Erre Systems (SELBACH) basiert, kommt de liche Bedeutung mehr zu. Einblick in Reaktionsabläufe können uns nach B Verlaufsbeobachtungen über längere Z mischen Reaktionsfähigkeit auf Belast auf die gewählte verabfolgte „Reizd fähigkeit des Organismus und der Rea von der Tonuslage, der Ausgangslage WEZLER).

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Proceedings of the Montana Academy of Sciences 28: 51-57, 1968

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EFFECT OF FATTY ACIDS ON *SHIGELLA**

Mitsuru Nakamura and Marilyn Jo Zangar**

The mechanism of pathogenicity in *Shigella* has not yet been clearly elucidated. A number of variables appear to be important; namely, the host, the age of the host, the size of the infective dose, the "strain" and species of the etiologic agent, and the normal intestinal flora. The role of the normal intestinal flora in *Shigella* infections has been reviewed recently (5, 6). There is general agreement that the normal bacterial flora protects the animal against infection and disease due to the *Shigellae*.

Several workers reported that the growth of *Shigella* was inhibited when grown in a mixed culture with other bacteria (1, 4). The associated bacteria apparently produced some chemical or physical change in the environment that affected the growth of the pathogen.

The mechanisms of these interacting reactions become of interest in order to establish the specific role of the normal flora. Hentges reported that *Klebsiella* inhibited *Shigella* in mixed cultures (2). He suggested that the formic and acetic acids produced by *Klebsiella* were responsible for the inhibition of the *Shigella*.

The present investigation was undertaken to study the effects of several fatty acids on the *in vitro* growth of *S. sonnei* and the related species *Escherichia coli* as well as the enteropathogenic variant of *E. coli*. The effects of acetic, propionic, and octanoic acids were studied on *E. coli* B, *E. coli* OB E 0127:B8 (enteropathogenic), *S. sonnei* strain 9453, and *S. sonnei* strain 6761. The bacteria were maintained and exposed to fatty acids in the chemically defined medium (2). The pH of the media containing various concentrations of fatty acids ranging from 0 to 1.8 percent was adjusted to 7.0.

Standard plate counting techniques were used to enumerate the bacterial populations after incubation for 24 hours at 37° C. The activity of the fatty acids on the growth of

* This investigation was supported in part by a research grant AI-07668-01, National Institutes of Health, U. S. Public Health Service.

** Undergraduate research participant in microbiology supported by grant GY-2547, Undergraduate Education in Science, National Science Foundation.

Caprylic Acid

E. coli B is illustrated in Figure 1. Octanoic acid was highly toxic and a concentration of 0.3 percent essentially sterilized the culture. Acetic and propionic acids, after producing an initial partial inhibition, did not significantly reduce the growth of this organism even at 1.5 to 1.8 percent. Octanoic acid was the most toxic fatty acid for *E. coli* OB E 0127:B8 (Fig. 2). Acetic and propionic acids were less inhibitory to this strain.

The dose-response curves in Figure 3 and Figure 4 illustrate the activities of the fatty acids against *S. sonnei* strains 9453 and 6761. Octanoic acid was the most bactericidal of the fatty acids. Propionic acid was inhibitory to both strains, although there was a slight difference in the rate of inhibition. The lethal effect of propionic acid was exponential. Acetic acid was highly toxic to strain 6761 but relatively nontoxic to strain 9453 of *S. sonnei*. This is interpreted as due to strain difference in susceptibility to acetic acid.

The three fatty acids studied exerted different inhibitory patterns on the related bacteria studied. According to Hentges the inhibitory activity of acetic acid cannot be attributed to a hydrogen ion effect alone (3). However, the mechanisms by which these fatty acids exert their antibacterial effect are not understood.

Hentges has hypothesized that the fatty acids, particularly acetic acid, are inhibitory for *Shigella* in the intestine (2). It is possible that the fatty acids produced in the intestine by the normal flora inhibit the *Shigella* and thus reduce the possibilities of disease following infection. In view of the fact that our results suggest a considerable variation in susceptibility of *S. sonnei* to acetic acid, we would like to suggest that other mechanisms may operate, besides fatty acid toxicity, in the interaction between *Shigella* and the normal intestinal flora. Furthermore, the acetic acid produced in the intestine will also inhibit *E. coli*, resulting in a much more complex interacting system than merely the inhibition of *Shigella* by the products of another organism.

Currently we are studying the effects of other fatty acids on *Shigella*. We plan to study the effects of fatty acids on *Shigella-Escherichia* hybrids in order to determine if there are phylogenetic relationships among these organisms in relation to fatty acid sensitivity.

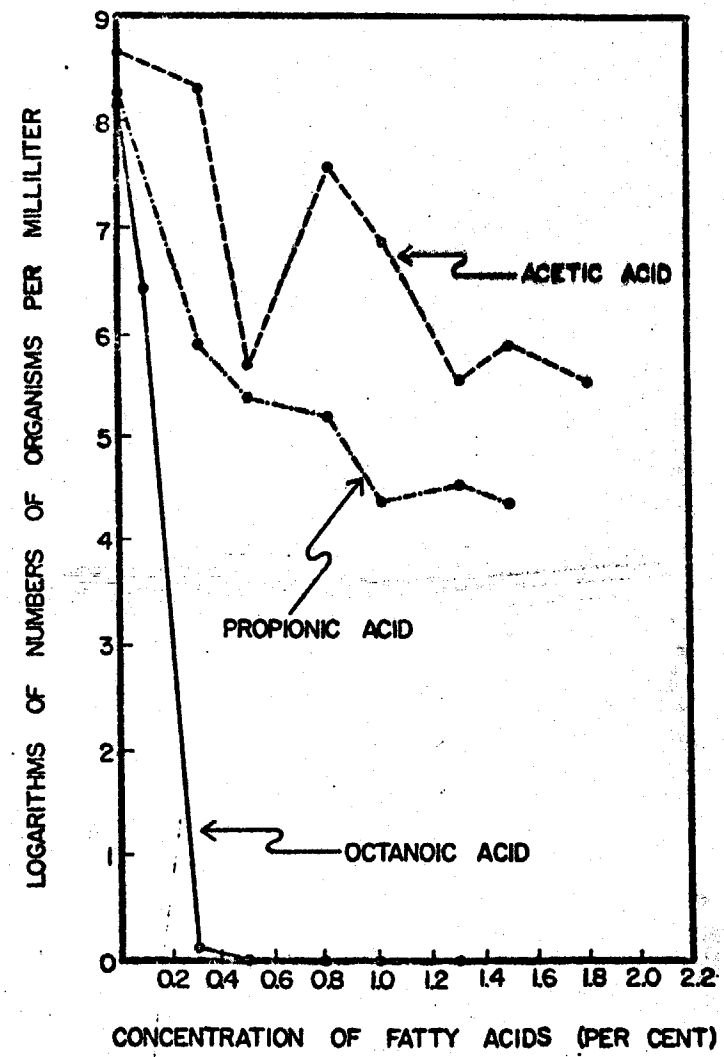

Figure 1. Effect of Acetic, Propionic, and Octanoic Acids on *Escherichia coli* B.

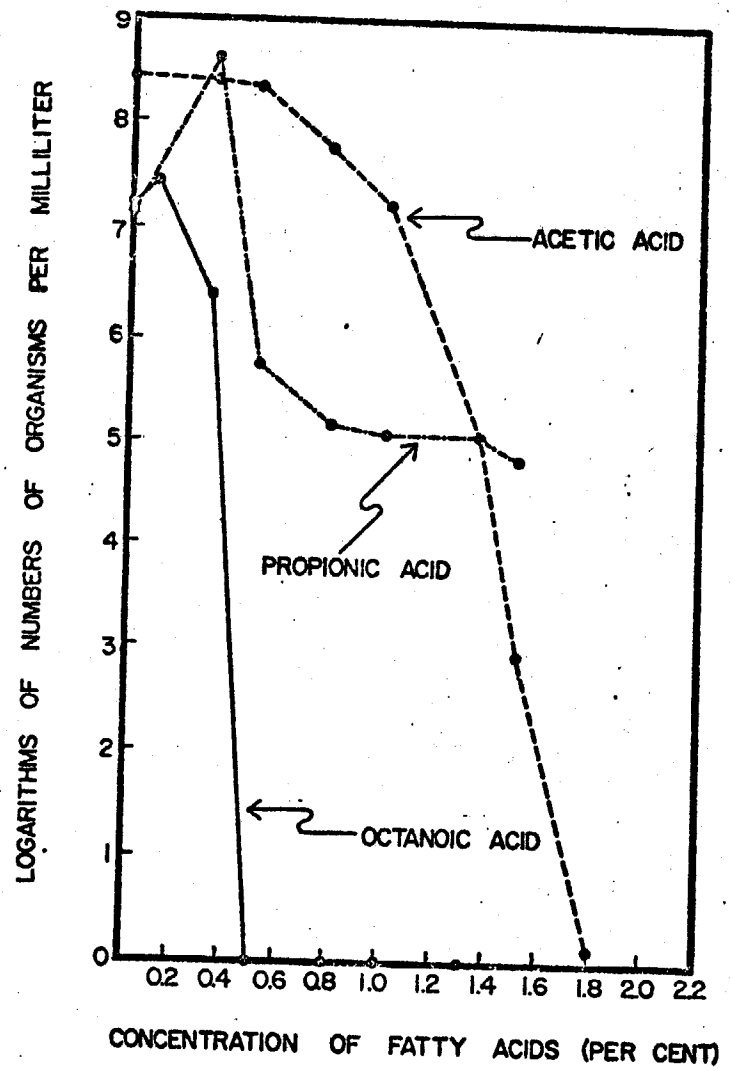

Figure 2. Effect of Acetic, Propionic, and Octanoic Acids on *Escherichia coli* OB E 0127:B8.

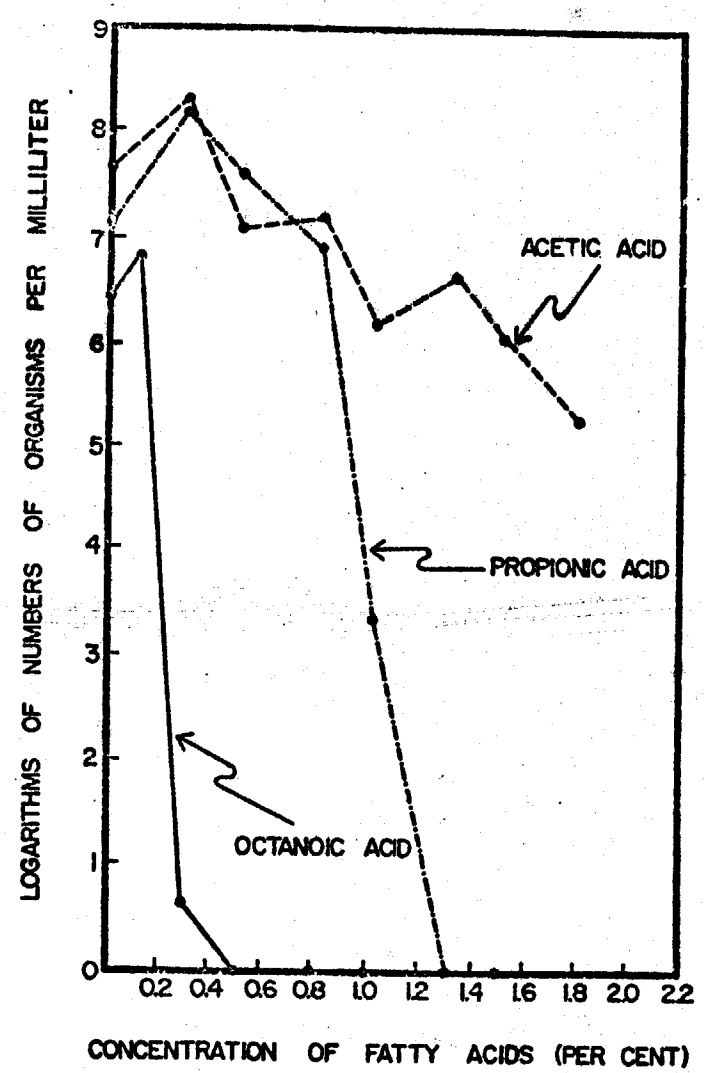

Figure 3. Effect of Acetic, Propionic, and Octanoic Acids on *Shigella sonnei* 9453.

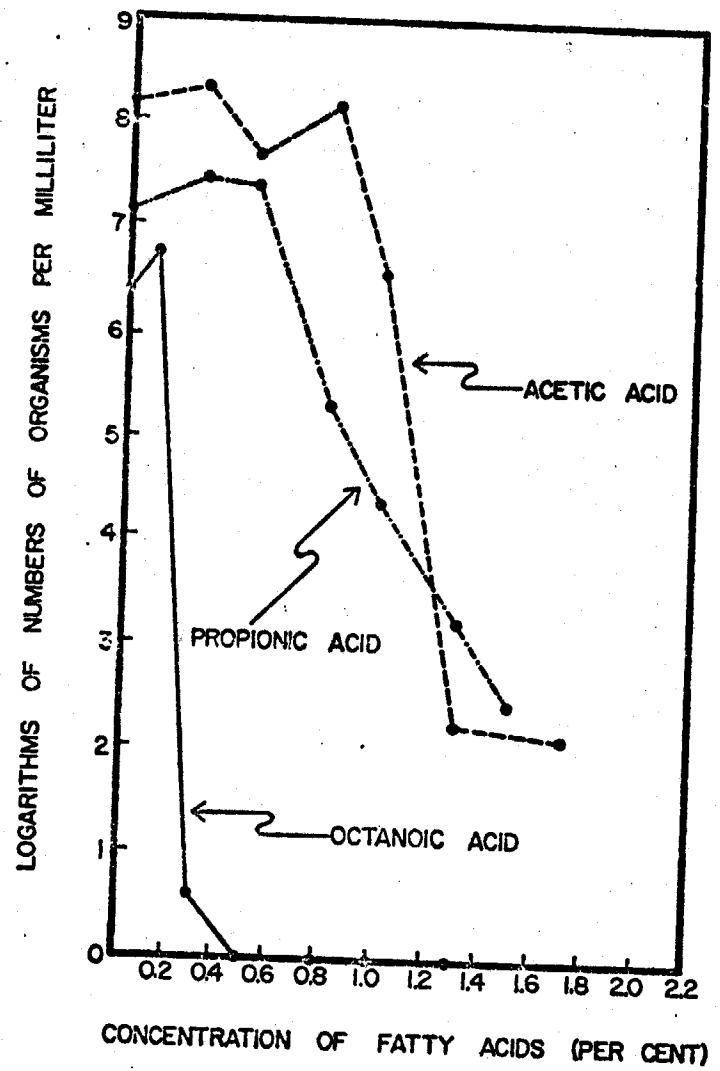

Figure 4. Effect of Acetic, Propionic, and Octanoic Acids on *Shigella sonnei* 6761.

Montana Academy of Sciences 28: 51-57, 1968

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Diabetes 18(2): 96-100 (1969)

Insulin and Ketone Responses to Ingestion of Medium and Long-chain Triglycerides in Man

F. Xavier Pi-Sunyer, M.D., Sami A. Hashim, M.D., and Theodore B. Van Itallie, M.D.,
with technical assistance of Eva deLooze

New York

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SUMMARY

The effects of medium chain triglyceride (MCT), corn oil, and water on serum glucose, ketones, and immunoreactive insulin (IRI) were compared. Fourteen normal subjects ingested 1 gm. per kg. of MCT (composed principally of C8 and C10 fatty acids) and on another occasion an equal volume of water. After water ingestion, glucose, ketones, and IRI did not change appreciably up to five hours. After MCT, a significant rise in serum ketones and IRI was associated with a slight fall in serum glucose. Fourteen normal subjects ingested 1 gm. per kg. corn oil, and on another occasion an equal volume of water. Serum glucose, ketone and IRI concentrations again did not change up to five hours after ingestion of water. After corn oil, no significant rise in ketones occurred. There was a small rise in IRI and a slight fall in glucose. In the eight subjects in whom MCT, corn oil and water were compared a certain degree of insulin secretory response was obtained after MCT and corn oil but not after water ingestion. However, the IRI response after MCT was highest. The rise in IRI without a corresponding change in serum ketones after ingestion of the long chain triglyceride might be attributed to a gastrointestinal β-cytotropic effect. The mechanism of IRI stimulation by MCT also has been related to MCT-induced hyperketonemia and possibly to a direct effect on insulin secretion of small amounts of octanoate in peripheral blood. DIABETES *18*:96-100, February, 1969.

The hypoglycemia induced by administration of acetoacetate and other ketones has been ascribed to an insulin stimulatory effect.[1] The degree of hyperketonemia at which this stimulatory effect occurs has not been clarified. The ingestion of medium chain triglycerides can induce a modest hyperketonemia in man.[2,3] The purpose of the present study was to ascertain the insulin secretory response to medium chain triglyceride-induced hyperketonemia in normal human subjects. Also, control experiments were conducted to estimate the insulin secretory response of subjects to the ingestion of a long chain triglyceride or simply water.

MATERIALS AND METHODS

Initially, fourteen normal subjects (ten men and four women) were studied on two separate mornings at least one week apart. All subjects were lean, healthy volunteers, eighteen to thirty-five years of age. Following overnight fast, each individual ingested either 1 gm. per kg. body weight of medium chain triglyceride (MCT) or an equivalent volume of water. The fatty acid composition of the MCT preparation was approximately 80 per cent octanoate (C8) and 17 per cent decanoate (C10) with traces of laurate (C12) and hexanoate (C6).[4]

Additional studies were made by feeding volunteers 1 gm. per kg. body weight of long chain triglyceride (corn oil). Eight of the original volunteers and six new ones were available for this purpose. The MCT or the corn oil was emulsified by ultrasonication in an equal amount of water containing 0.5 ml. of Tween 80. The same amount of Tween 80 was added to the water control "meal." Venous blood samples were obtained before and for five hours after the ingestion of either the fat or the water. After the blood was permitted to clot on ice for three hours, the serum was separated and immediately frozen until use. The serum samples were analyzed for glucose,[5,6] total ketones,[7,8] and immunoreactive insulin.[9,10] The immunoreactive insulin (IRI) was measured against a human insulin standard.* Duplicate values of the per cent activity of the bound insulin precipitates had to agree within 3 per cent to be considered valid.

From the Department of Medicine, St. Luke's Hospital Center and the Institute of Nutrition Sciences, Columbia University, New York, New York.

*Kindly supplied by Dr. Mary Root, Lilly Laboratories, Indianapolis, Indiana.

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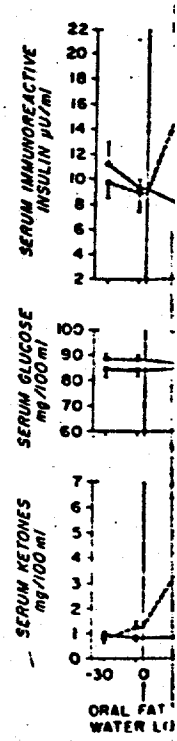

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RESULTS

The results obtained in the fourteen subjects who took MCT or water are shown in figure 1. In presentation of the results, all values represent means unless otherwise specified, those described as "baseline" represent the average of the pre-ingestion means measured at —30 and —5 min. The baseline postabsorptive serum ketone levels in the fourteen subjects studied were 0.77 ± 0.26 (S.E.) mg. per 100 ml. After water ingestion serum ketones remained at a level below 1.3 mg. per 100 ml. After MCT serum ketones rose to a peak, however, of 6.7 mg. per 100 ml. at two hours, and then were maintained at a level above 6.0 mg. per 100 ml. for five hours. Serum insulin concentrations ranged from 6.6 to 11. 2 μU. per ml. throughout the water ingestion period, the average of these means being 8.2 μU. per ml. After MCT insulin rose from a baseline level of 9.3 μU. per ml. to values of 17.7 μU. per ml. at thirty minutes ($p < .005$), 15.3 μU. per ml. at one hour ($p < .005$) and 15.4 μU. per ml. at two hours ($p < .05$). Values at four and five hours were elevated above the baseline value, but the differences were not significant. Serum glucose remained constant at about 85 mg. per 100 ml. before and after water ingestion. After MCT there was a slight fall in serum glucose values varying between 6 and 8 mg. per 100 ml. which, after one hour, were significantly lower ($p < .005$) than the preingestion values.

The results in the fourteen volunteers who ingested corn oil and then water are shown in figure 2. Serum ketones did not change significantly after water ingestion, remaining at a level below 1.2 mg. per 100 ml. After corn oil, ketones rose very slightly with the highest level of 2.0 mg. per 100 ml. recorded at five hours. Serum insulin concentrations after water did not rise but actually fell slightly below the baseline level of 10.4 μU. per ml. After corn oil, insulin rose from a baseline concentration of 10.9 μU. per ml. to values of 12.7 μU. per ml. at thirty minutes, 13.4 μU. per ml. at one hour, 13.8 μU. per ml. at two hours, diminishing again to 11.9 μU. per ml. at four hours and 10.2 μU. per ml. at five hours. With the exception of the two-hour concentration ($p < .05$), these values did not differ significantly from the baseline level. Serum glucose did not change during water ingestion, while after corn oil there was a slight fall in glucose which, at sixty minutes, was lower than the pre-ingestion values ($p < .05$).

A comparison of results obtained after water, corn oil

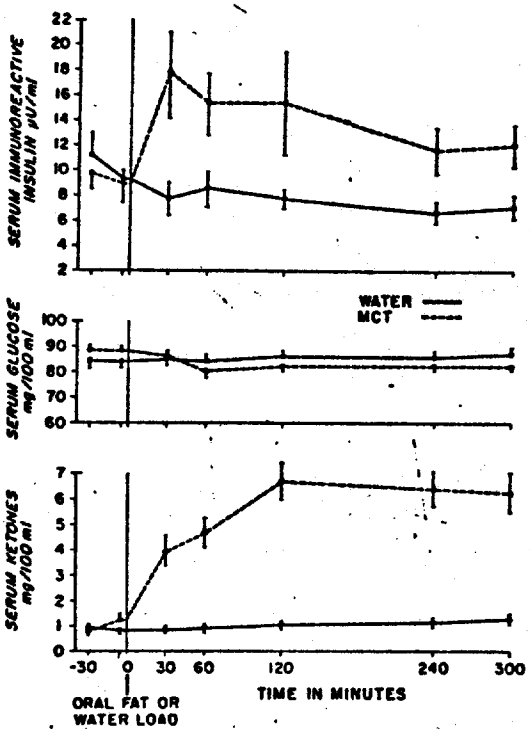

FIG. 1. Mean serum immunoreactive insulin, glucose, and ketone responses (± S.E.) to ingested water and MCT in fourteen normal subjects.

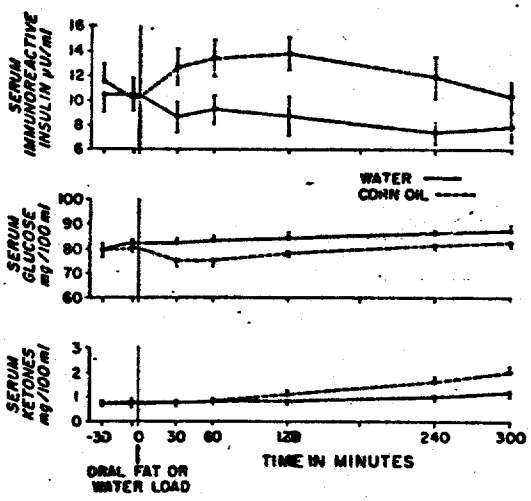

FIG. 2. Mean serum immunoreactive insulin, glucose, and ketone responses (± S.E.) to ingested water and corn oil in fourteen normal subjects.

and MCT in the eight subjects who underwent all three studies is shown in figure 3 and table 1. When the IRI responses to MCT were compared with those after water, there was a significantly ($p < 0.025$) higher IRI response throughout the period following ingestion of MCT. In contrast, when IRI responses to corn oil were compared with those obtained after water ingestion, significantly higher IRI responses were detected only at thirty and 120 minutes. When MCT was compared with corn oil, significantly higher IRI responses were obtained with MCT at thirty minutes.

DISCUSSION

MCT has been shown previously to induce modest hyperketonemia in man.[2,3,11] This phenomenon has been related to the perfusion of the liver by medium chain fatty acids which travel in the portal vein after digestion and absorption of the fatty acids and monoglyceride derivatives of the parent triglyceride.

The hypoglycemic action of ketone bodies infused into animals has been reported by a number of investigators.[12,13] The hypoglycemia induced by ketone administration in portacaval shunted dogs by Mebane and Madison[14] was ascribed entirely to a reduction in hepatic glucose output. Subsequently, Madison et al.[1]

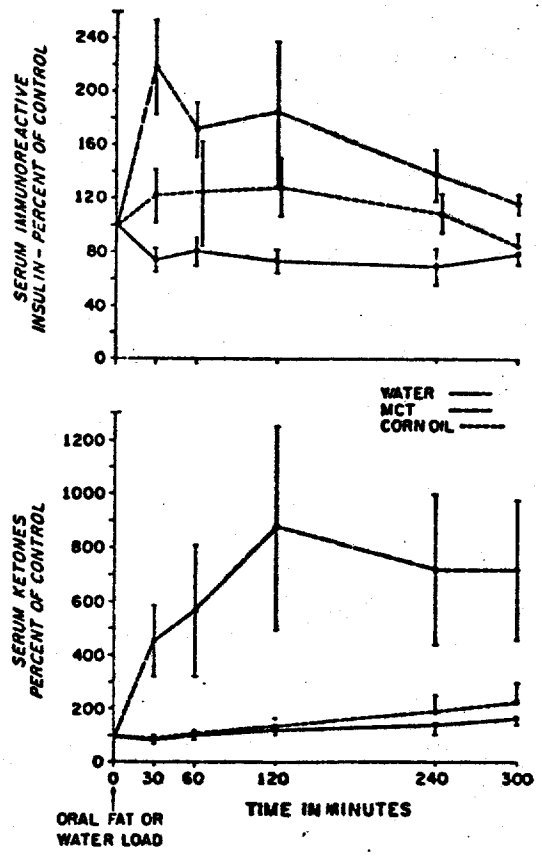

FIG. 3. Mean serum immunoreactive insulin and ketone responses (per cent of control ± S.E.) to water, corn oil, and MCT ingestion in eight normal subjects.

TABLE 1

Mean serum immunoreactive insulin (IRI), glucose, and ketone responses (± S.E.) to water, corn oil, and MCT ingestion in eight normal subjects. Values in parentheses denote per cent of control, with 100 per cent being the average of the two pre-ingestion values at —30 and —5 minutes.

Time in minutes		−30	−5	+30	+60	+120	+240	+300
	IRI	10.5±2.6	8.7±1.8	7.1±1.6 (74)	7.8±1.5 (81)	7.0±1.2 (73)	6.6±1.2 (69)	7.5±1.7 (78)
Water	Glucose	83±2.8	85±2.3	84±3.1 (100)	85±2.9 (101)	86±3.8 (102)	88±2.8 (105)	89±3.4 (106)
	Ketones	0.9±0.2	0.7±0.2	0.7±0.1 (88)	0.8±0.2 (103)	0.9±0.2 (120)	1.1±0.2 (136)	1.3±0.3 (161)
	IRI	10.9±2.3	10.1±1.7	12.8±2.5 (122)	13.0±2.5 (124)	13.3±1.5 (127)	11.4±2.7 (109)	8.9±1.7 (85)
Corn oil	Glucose	82±3.6	82±2.3	77±3.9 (94)	76±3.4 (93)	79±2.5 (96)	82±2.6 (100)	83±2.8 (101)
	Ketones	0.8±0.2	0.9±0.3	0.8±0.2 (87)	0.9±0.2 (105)	1.1±0.2 (129)	1.6±0.2 (185)	1.9±0.2 (221)
	IRI	10.7±2.1	8.4±1.6	20.8±6.5 (218)	16.4±4.5 (172)	17.6±7.2 (184)	13.1±2.8 (137)	11.1±2.2 (116)
MCT	Glucose	87±3.6	87±1.3	85±2.0 (98)	78±2.2 (90)	83±2.5 (95)	81±2.4 (93)	82±1.6 (94)
	Ketones	0.8±0.2	1.0±0.2	4.1±0.7 (450)	5.1±0.9 (561)	7.9±0.9 (870)	6.6±0.9 (712)	6.5±1.1 (718)

demonstrated enhanced insulin levels in pancreatic venous blood after ketone infusions, but these workers could not obtain a similar hypoglycemia in depancreatized dogs. The suggestion was made that the modestly increased insulin levels induced by hyperketonemia dampen further ketone production by regulating free fatty acid release from adipose tissue thereby preventing severe ketoacidosis during starvation. Since no direct ketone measurements were made by Madison et al., the degree of ketonemia at which an insulin stimulatory effect would occur was left undefined, albeit these authors estimated that an insulin secretory effect occurred at levels of 3.5 mMoles per liter (approximately 21 mg. per 100 ml. of acetone equivalent)

In the present experiments, the modest hyperketonemia induced by MCT was associated with a small rise in circulating IRI. Although serum IRI concentrations of 18 μU. per ml. would be considered within the normal postabsorptive range, the changes that occurred after MCT were not evident after water. The change in serum IRI in response to MCT may be related in part to the hyperketonemia. This interpretation is consistent with the studies in dogs by Madison et al.[1] However, Fajans et al.[15] attained plasma ketone values of 25 mg. per 100 ml. after infusing acetoacetate into normal volunteers and were unable to detect a rise in plasma IRI. Moreover, infusion of β-hydroxybutyrate into man has not been found by others[16,17] to enhance insulin secretion.

The possibility that octanoate is capable of directly stimulating release of insulin by the beta cells of the pancreas should be considered. Sanbar et al.[18] infused octanoate and found a slight increase in peripheral plasma IRI in three of the six dogs studied. Other studies of infusion of octanoate in dogs have not shown enhanced insulin secretion.[19] In any event, these studies involve direct introduction of octanoate into the systemic circulation. Under the conditions of the present study, MCT ingestion results in transport of octanoate and decanoate into the portal vein and the liver where these acids are extensively metabolized.[20] Thus, the ingestion of MCT is not associated with an appreciable rise of MCT-derived acids in the systemic circulation.[21] Whether the small rise in arterial blood octanoate that might occur after MCT ingestion could directly stimulate insulin secretion cannot be determined from the present study.

Since serum ketones exhibited virtually no rise after corn oil, it is unlikely that they played a role in the small rise in circulating IRI observed. However, the presence of ingested fat in the gastrointestinal tract may result in the release of certain intestinal hormones. Some of these hormones have been shown by Unger et al.[22] to stimulate insulin secretion. Thus, it is possible that the enhancement of insulin secretion in response to corn oil ingestion may be ascribed to a direct effect of the fat load on the gastrointestinal tract with release of β-cytotropic hormones. However, direct evidence for this possibility is not provided by the present study.

When the IRI responses were compared after MCT, corn oil, and water in the same eight subjects, it was evident that MCT induced the highest insulin response. Thus, the mechanism of IRI stimulation by MCT must be related to an effect other than a possible fat-mediated gastrointestinal hormonal release. Whether the higher degree of insulin response to MCT ingestion is induced by the hyperketonemia or the small amount of octanoate that may have escaped hepatic metabolism cannot be determined from the present study.

ACKNOWLEDGMENT

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Dr. Pi-Sunyer is a Fellow of the New York Heart Association and Dr. Hashim is Career Development Investigator, National Heart Institute.

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Body Odor and Metabolic Defects

...N-Butyric/N-hexanoic acidemia is another rare condition in which the exhaled air, tissues, and body fluids have the strong odor of sweaty feet. This condition has so far been observed in two families, involving four and possibly five infants (J. B. Sidbury, Jr., W. R. Harlan, and B. Wittels, Am. J. Dis. Child. *104*:531, 1962; Sidbury, E. K. Smith, and Harlan, J. Pediat. 70:8, 1967). This abnormality of short chain fatty acid metabolism, in which the odor is caused by butyric and hexanoic acids, is probably an autosomal recessive disorder and is due to a deficiency of the acyl dehydrogenase specific for the metabolism of four and six chain fatty acids. Feeding difficulties, lethargy, weakness, and odor developed in these children in the first days of life and were followed by severe acidosis, dehydration, and convulsions reminiscent of tetany. Death ensued in the first month from bone marrow depression with pancytopenia, a bleeding diathesis, and gram negative sepsis.

Other rare disorders which must be considered when an infant has a peculiar odor include the oasthouse syndrome, hypermethioninemia, and methionine malabsorption.

The child with oasthouse syndrome described by A. J. Smith and L. B. Strang (Arch. Dis. Child. *33*:109, 1958), had a distinctive unpleasant pungent smell reminiscent of a brewery or oasthouse. The clinical pattern was like that of phenylpyruvic oligophrenia, but differed in unresponsiveness to stimuli, muscular hypotonicity, recurrent episodes of fever, tachypnea, and edema. White hair grew at nine months and death occurred at ten months of age. A double metabolic defect existed, since phenylketonuria was also present. The smell of the urine differed from the mousy odor of phenylacetic acid, which is commonly excreted in phenylketonuria, and was thought due to an oxidation or polymerization product of alpha-hydroxybutyric acid....

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THROMBOTIC, ATHEROSCLEROTIC AND LIPEMIC EFFECTS OF DIETARY FATS IN THE RAT

SERGE RENAUD, V.M.D., PH.D.*

A few years ago, Pickering[1] noted, "The great weakness of the lipid hypothesis is that it does not account for the thrombus." He did not consider as satisfying to a clinician that alterations in lipid metabolism could also predispose to thrombosis. Nevertheless, numerous studies have indicated a relationship between thrombosis and hyperlipemia,[2-4] as well as the importance of lipids in blood clotting[5]; in addition, there is a strong possibility that the long-chain saturated fatty acids of the dietary fat are responsible for thrombosis[6] being by far the most effective fatty acids in aggregating blood platelets *in vitro*.[7, 8]

In recent experiments in rats, we devised an experimental model[9] that results in a constant and extremely elevated incidence of easily observed large occlusive thromboses, provided the animals are fed certain high-fat diets for 6 to 10 weeks.[10] Thrombosis is then initiated by the intravenous injection of a Gram-negative bacteria endotoxin, resulting in multiple, red hepatic infarcts, and a simple quantitative evaluation of the phenomenon can be achieved merely by weighing the lesions, as will be described in this paper.

By adding fatty acids to the basic high-fat diet, we were able to show[11-13] that, under these conditions, as *in vitro*, the long-chain fatty acids were once again the most thrombogenic and that stearic acid appeared to be the most active among them. However, the very-long-chain fatty acids, such as behenic acids, were not thrombogenic, probably because of very poor digestibility.[12, 13]

In these studies in rats, as in man,[14, 15] palmitic acid was the most hypercholesterolemic of the fatty acids used, whereas stearic acid or short-chain fatty acids such as caprylic acid were much less hyperlipemic. As was also shown in man,[16] only linoleic and linolenic acids, but not oleic acid, were hypolipemic.

Other investigators working on the relationship between the human diet and its lipemic effects[15] recently suggested that people wishing to avoid coronary disease should substitute cacao butter for butter in the diet, because the former is richer in stearic acid, a long-chain saturated fatty acid that is much less hypercholesterolemic than palmitic, myristic and lauric acids. Judging from the results of work with blood platelets *in vitro*, such recommendations could be disastrous for coronary patients.

Inasmuch as our previous findings with dietary fatty acids seemed to have established the essential link between lipemia in man and the work with

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blood platelets and coagulation *in vitro*,[12, 13] it was deemed necessary, because of the obvious implications, to make a detailed study under our experimental conditions of most of the common fats utilized in human nutrition. Our aims in these experiments were to verify: (1) whether the thrombogenic, atherogenic and lipemic effects of a dietary fat could be predicted from its composition in fatty acids; (2) to what extent the degree of lipemia as expressed by the serum cholesterol or triglyceride level could be a reliable indication of the thrombogenic or atherogenic capacity of a fat.

In addition, since we had not previously studied myristic and lauric acids, and a knowledge of their effect under our experimental conditions was essential for the present work on dietary fats, these fatty acids were studied here in a small experiment in comparison with caprylic, palmitic and stearic acids.

MATERIAL AND METHODS

Male Holtzman rats with an initial body weight of 150 to 170 gm were utilized. For each experiment, the exact number of animals actually utilized is shown in the corresponding table. The results of experiment 2 (table 2) and experiment 3 (table 4) represent the pooled data of several sets of experiments, with 6 animals per group in each set. These data were pooled because they were practically identical and resulted from experiments performed at approximately the same time and under comparable conditions. The rats were housed in a constant temperature environment and given, *ad libitum*, tap water and the hyperlipemic diet described in detail elsewhere.[10, 11] In the tables are listed the ingredients composing the diets utilized in the corresponding experiment. The casein, cellulose (Alphacel), cholesterol, salt mixture (Wesson), sodium cholate, sucrose and vitamin mixture (vitamin diet fortification mixture) were obtained mixed in the right proportion by the supplier. The fatty acids utilized in experiment 1 (table 1) were also purchased from the same source (Nutritional Biochemicals Corporation). The animal fats utilized were ground and then melted in a steam bath; a sample of each of these tallows was analyzed by gas-liquid chromatography and the results are reported in table 3. Except for the coconut oil (Matheson, Coleman and Bell) and cacao butter, kindly supplied by Fry-Cadbury Ltd., all the fats and oils were commercially available and were therefore purchased from local stores. When the diets were prepared, isocaloric amounts of the fat (melted when necessary) were carefully mixed with the basic ingredients and then kept under refrigeration for not more than 2 weeks.

At the end of the feeding periods and following 17 hr of fasting, approximately 3 ml of blood were removed in siliconized syringes, equipped with 20-gauge needles, from the jugular vein of the rats, under light ether anesthesia, as reported in detail elsewhere.[11] To initiate thrombosis in experiments 1, 2 and 3 (tables 1, 3 and 4), a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide (0.3 mg per kg) in saline was injected by the same needle, after the last withdrawal of blood.

In each rat of the first three experiments, the plasma clotting time after recalcification was performed in duplicate, in plastic tubes (polycarbonate No. 2804, International Equipment Company, Boston), according to the technique already reported.[11] Triglycerides and total cholesterol analysis were performed on individual sera. The triglycerides were determined by the Van Handel method.[17] In experiment 3 (table 4), Florisil (60 to 100 mesh, Fisher Scientific Company) was used, instead of zeolite as suggested by other investigators[18], but chloroform was still kept as the extracting fluid. Only 0.3 ml of serum was utilized for this determination. The total serum cholesterol was determined by a manual adaptation of the Technicon Auto-Analyzer automated ferric chloride technique,[19] which yielded remarkably reproducible results. In brief, 0.2 ml of serum was added to 4.8 ml of isopropanol, carefully agitated and, after standing for 15 min., centrifuged for 5 min. To 0.5 ml of the supernatant were first added 4 ml of a 0.05 per cent ferric chloride solution in 95 per cent acetic acid and then 3 ml of concentrated sulfuric acid. After 5 min. of centrifugation, the reading was made at 550 mμ against a blank.

On 6 rats in each group of experiment 3 (table 4), no serum determinations were carried out; instead, heparinized plasma was collected on ice to determine the fibrinogen, according to the method of Goodwin[20] utilizing only 0.5 ml of plasma, and the unesterified fatty acids by the technique of Itaya.[21]

In the thrombosis experiments, the time of death was recorded and the animals were autopsied. Survivals were killed at 24 hr. with chloroform. Except in experiment 2 (table 2), in which the lesions were graded according to a scale of 0 to 3 as described elsewhere,[13] a quantitative evaluation of the thrombotic phenomenon could be obtained by weighing the liver before and after removing the red infarcts, as shown in figure 1. The severity of the thrombotic phenomenon was then expressed as the percentage in weight of infarcted liver.

In the atherosclerosis experiment (table 5), the animals were killed with chloroform after the last blood removal and the heart and the aorta were dissected as far as the common iliac arteries. The severity of the aortic fatty streaks was then graded according to a scale of 0 to 3 as already described,[13] under binocular examination of the fresh aorta, opened longitudinally. Macroscopic readings were verified by routine histologic examination of the heart and aorta.

RESULTS

Experiment 1 (table 1). Depending on the fatty acid added to the already high fat diet, the results obtained differed markedly. Palmitic acid gave rise to by far the highest values for cholesterol and triglycerides; the next most hyperlipemic fatty acids were caprylic and myristic acids, followed by stearic acid; lauric acid was the least hyperlipemic. In group 5 fed stearic acid, the plasma clotting time was the shortest and the severity of thrombosis the highest; this was followed by the results for group 4 in which the clotting time was significantly longer and the thrombotic lesions were less severe, although not

TABLE 1

Experiment 1: thrombogenicity of various fatty acids

Results = mean ± S.E. Duration of the experiment, 6 weeks. Diet composition in addition to the fatty acid (5 per cent) listed below: butter 32, casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2, water 1 (weight per cent).

Group and Fatty Acid	No. of Carbon Atoms	No. of Animals	Cholesterol	Triglycerides	Plasma Clotting Time	Thrombosis (Severity)	Survival
			mg/100 ml		*sec.*		*hr.*
1. Caprylic...	8	7	720 ± 172	156 ± 24	146 ± 8	2.0 ± 1.1	9.3 ± 2.3
2. Lauric.....	12	6	401 ± 33	111 ± 29	198 ± 17	2.3 ± 1.6	12.7 ± 3.4
3. Myristic...	14	7	738 ± 37	154 ± 16	181 ± 9	0	7.4 ± 2.8
4. Palmitic...	16	7	1153 ± 135	258 ± 30	143 ± 8	8.2 ± 3.6	3.3 ± 1.5
5. Stearic.....	18	7	528 ± 74	180 ± 15	121 ± 3	14.5 ± 0.3	11.4 ± 2.5

significantly so. The thromboses in the groups fed caprylic, lauric and myristic acids were much less severe, but only in groups 2 and 3 was the clotting time prolonged. The survival time was shortest in group 4, where the highest lipemia was seen, and longest in groups 2 and 5, where the lowest cholesterol and triglyceride levels were observed.

Experiment 2 (table 2). From experiment 1, it could be concluded that stearic acid was the most thrombogenic of the fatty acids studied. Therefore, if these results were to have practical applications for dietary fats, the fats richest in stearic acid should be the most thrombogenic. Table 3 shows the composition of various common fats differing in the type of fatty acids they contain, particularly in the quantity of stearic acid. From this table it can be seen that cacao butter contains 35 per cent stearic acid, butter 14.6 per cent, lard 7.8 per cent and corn oil 3.4 per cent.

As is seen in table 2, after only 8 weeks of dietary feeding, the severity of thrombosis of the butter-fed animals was 0.6 and the mortality rate was 30 per cent. The animals fed cacao butter presented a shorter clotting time, a severity of thrombosis of 2.1, and a 100 per cent mortality rate. Here, however, with cacao butter as the only fat, cholesterol and triglycerides were higher than with butter. After 10 weeks of dietary feeding, the severity of thrombosis in group 3 fed butter was 1.9. The serum cholesterol and triglycerides were no higher than after 8 weeks, but the clotting time was considerably decreased from 185 to 139 seconds. In group 4 fed lard, the severity of thrombosis was only 0.8; the cholesterol and triglycerides were lower and the clotting time longer than in group 3. The mere fact of reducing the butter content to 32 per cent and substituting 6 per cent of corn oil (group 5) markedly prolonged the clotting time and reduced the lipemia; the severity of thrombosis was only 0.4. When corn oil was the only fat in the diet (group 6), the triglycerides, but not the cholesterol, showed a further decrease as compared with group 5; the clotting time was prolonged and the severity of thrombosis decreased to 0.1.

TABLE 2

Experiment 2: thrombogenicity of various fats

Results = mean ± S.E. Diet composition in addition to the ingredients listed below: casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Group and Duration	No. of Animals	Cholesterol	Triglyceride	Plasma Clotting Time	Thrombosis (Severity, 0-3)	Mortality	Survival
		mg/100 ml		*sec.*		*%*	*hr.*
8 weeks							
1. Cacao butter 32 + water 6....	23	830 ± 23	148 ± 6	155 ± 7	2.1	100	4.2 ± 0.7
2. Butter 38.....	12	585 ± 80	135 ± 4	185 ± 12	0.6	30	18.1 ± 2.3
10 weeks							
3. Butter.....	35	487 ± 45	151 ± 12	139 ± 4	1.9	50	13.6 ± 1.6
4. Lard 32 + water 6.....	12	459 ± 25	115 ± 6	148 ± 8	0.8	58	14.2 ± 2.7
5. Butter 32 + corn oil 6.....	24	340 ± 21	91 ± 9	161 ± 11	0.4	46	16.7 ± 1.8
6. Corn oil 32 ± water 6.....	12	366 ± 1	75 ± 4	185 ± 7	0.1	16	23.0 ± 0.7

TABLE 3

Composition of certain fats in the commonest fatty acids

Selected values adapted from, "Fatty acids in animal and plant products," by V. R. Goddard and L. Goodall, Human Nutrition Research Division, United States Department of Agriculture, 1959.

Fatty Acids (Carbon Atoms)	Coconut Oil	Butter*	Cacao Butter	Bovine Tallow*	Lard	Chicken Tallow*	Horse Tallow*	Olive Oil	Arachis Oil	Corn Oil
Lauric (12).....	45.4	1.1								
Myristic (14).....	18.0	9.1		3.3		1.0	5.6			0.1
Palmitic (16).....	10.5	28.6	24.4	24.9	32.2	24.6	29.6	9.2	8.2	8.3
Stearic (18).....	2.3	14.6	35.0	16.9	7.8	6.9	4.9	2.0	6.2	3.4
Arachidic (20).....	0.4							0.2	4.0	0.4
Lignoceric (24).....									3.9	0.2
Palmitoleic (16:2H)...	1.3	2.7		5.3		6.5	6.0			1.2
Oleic (18:2H).....	7.1	33.0	28.2	46.3	48.0	45.3	31.3	83.1	52.9	30.0
Linoleic (18:4H).....	0.4	2.4	2.4	3.3	11.0	15.7	8.2	3.9	24.7	54.1
Linolenic (18:6H)....		1.4			0.6		15.0			0.6
Iodine index.....	7.8	33.4	37.0	48.3	64.0	79.3	82.7	84.4	90.1	126.7

* Determined in the laboratory of Professor J. M. Demers, Biological Sciences, University of Montreal.

Experiment 3 (table 4). In this study, the results of four different experiments that gave almost identical results were pooled. In table 4 are reported all the data obtained in these experiments; the different fats are listed in order of their decreasing thrombogenic capacity. Although all of the determinations were performed under rigid control conditions by the most reliable methods found to be compatible with the handling of a large number of samples by well-trained technicians, none of the parameters examined seem to have any connection with the severity of thrombosis, and only the survival time appears to be related to the triglyceride level. Figure 2 shows a correlation ($r = -0.81$)

TABLE 4

Experiment 3: thrombogenicity of various fats

Results = Mean ± S.E. Total duration of the experiment = 9 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water): casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Group and Fat	Iodine Index	No. of Animals	Cholesterol	Triglycerides	Unesterified Fatty Acids	Fibrinogen	Plasma Clotting Time	Thrombosis (Severity)	Survival
			mg/100 ml		*μmoles/l*	*mg/100 ml*	*sec.*		*hr.*
1. Butter	33.4	50	693 ± 83	192 ± 22	650 ± 20	350 ± 20	130 ± 2	16.2 ± 2.1	6.8 ± 1.0
2. Arachis oil	91.8	13	720 ± 50	75 ± 9			160 ± 7	9.4 ± 2.5	18.3 ± 1.7
3. Coconut oil	7.8	25	685 ± 37	125 ± 6	500 ± 50	340 ± 10	164 ± 7	4.8 ± 1.7	10.8 ± 1.8
4. Horse tallow	82.7	25	234 ± 29	45 ± 4	420 ± 10	420 ± 10	131 ± 6	4.8 ± 1.3	16.6 ± 1.7
5. Chicken tallow	79.3	28	450 ± 40	80 ± 8	460 ± 30	390 ± 10	141 ± 4	3.7 ± 0.9	16.7 ± 1.4
6. Olive oil	84.4	23	1022 ± 101	132 ± 13	370 ± 40	400 ± 20	144 ± 4	0.9 ± 0.4	11.1 ± 2.0
7. Bovine tallow	48.3	28	1016 ± 100	189 ± 19	460 ± 20	450 ± 40	165 ± 6	0.8 ± 0.4	9.3 ± 1.4

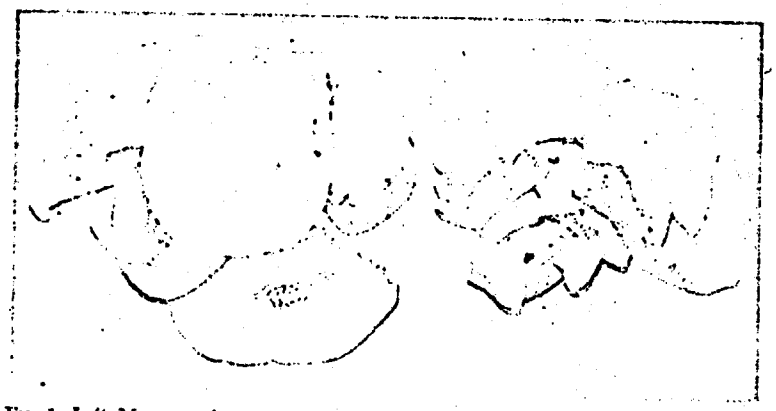

FIG. 1. *Left.* Macroscopic appearance of liver of rat fed high-fat diet, showing multiple red infarcts 3 to 12 hr. after the endotoxin injection. *Right.* A similar liver in which the infarcted areas have been removed. The difference in the weight of the liver before and after removal of the lesions gives a fair indication of the severity of the thrombotic phenomenon.

on a group basis between the serum triglycerides and the survival time on the pooled data of experiments 1, 2 and 3. The survival time was also related to the serum cholesterol, but the correlation coefficient r was only -0.69.

Experiment 4 (table 5). As in experiment 2, the highest serum cholesterol triglyceride level at both the 10- and the 18-week period was observed in rats fed cacao butter; in these animals, the most severe atherosclerotic lesions were also noted. In contrast to this, the group fed olive oil presented, at both periods, a degree of lipemia comparable to the butter-fed rats, but the lesions were incomparably less severe in the rats fed olive oil. Corn oil, the fat with the lowest lipemic effect, was also the least atherogenic.

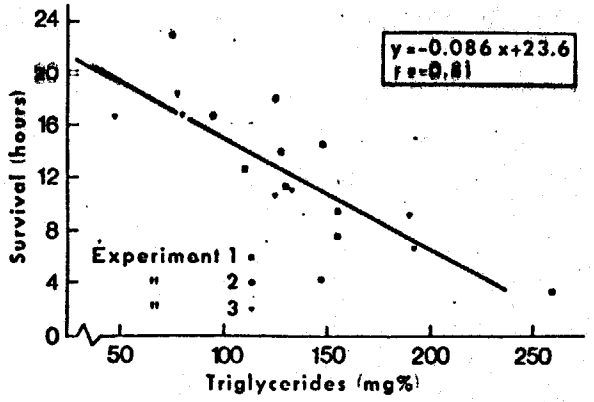

FIG. 2. The relationship, on a group basis, between the serum triglyceride level and the survival time in endotoxin-injected rats. The results are the pooled data of experiments 1, 2 and 3.

TABLE 5

Experiment 4: influence of dietary fats on lipemia and atherosclerosis

Results = Mean ± S.E. Total duration of the experiment 18 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water): casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Dietary Fat	No. of Autopsied Animals	Cholesterol		Triglycerides		Atherosclerosis (Scale 0-3)
		Week 10	Week 18	Week 10	Week 18	
		mg/100 ml		*mg/100 ml*		
Cacao butter	4	1136 ± 27	2775 ± 654	217 ± 14	582 ± 175	2.3
Butter	5	459 ± 11	2001 ± 696	124 ± 10	413 ± 160	1.7
Coconut oil	6	711 ± 9	516 ± 36	156 ± 25	117 ± 12	1.5
Olive oil	6	680 ± 203	1621 ± 342	188 ± 56	309 ± 70	0.3
Corn oil	6	301 ± 12	383 ± 34	57 ± 3	76 ± 9	0.05

DISCUSSION

The pathogenesis of our experimental model in which voluminous occlusive thromboses located almost exclusively in the hepatic veins are produced in rats fed a high-fat diet is not yet completely elucidated. Nevertheless, for years now, from one series of experiments to another, a continuously high incidence of multiple, red hepatic infarcts, resulting from the complete occlusion of the hepatic veins, has been consistently observed in rats fed the butter diet. We had already noted[9] that fatty infiltration of the liver has no bearing on the site of the thrombus formation. Despite the heavy fatty infiltration, it can also be added that liver protein synthesis in these animals is no different from that of rats fed laboratory chow.[22] In addition, we have observed in experiments not yet terminated that administration of epinephrine or norepinephrine instead of endotoxin to rats fed a high-fat diet will also initiate the formation of a voluminous thrombotic phenomenon, although in such cases the thrombi are located in the left atrium.

The most simple explanation for the hepatic veins as the primary site of thrombosis after an injection of Gram-negative bacteria endotoxin is that, in rat, the affinity of the liver for the bacterial toxins appears to be much greater than that of any other tissue.[23] It has also been observed[23] that, after the liver, the tissues retaining the largest quantity of toxins and the most frequent site of thrombosis are the spleen and the lungs. However, at the dosage of endotoxin utilized in this experiment, occlusive thrombi are found almost exclusively in the liver; therefore, from a practical point of view, only this organ has been considered in evaluating the severity of the thrombotic process.

The results of experiment 1 in this work confirm our previous results[12, 13] in that palmitic acid is much more hyperlipemic than stearic and caprylic acid, a finding that is also concordant with results obtained in man.[14, 16] In contrast to this, our observations with myristic and lauric acids are markedly different from those reported in man.[14, 16] Myristic acid induced a lipemia comparable to that induced by caprylic acid, and lauric acid was by far the least hyperlipemic of the five saturated fatty acids examined here. Of course, these results were obtained with purified free fatty acids and, as previously discussed,[13] the digestibility of the corresponding triglyceride can differ markedly from that of the acid and could explain the discrepancy in the results obtained when fats are used. However, among the fats studied in this series, only coconut oil contained large amounts of lauric and myristic acids. The results of experiment 3 showed that even in large-scale experiments, the cholesterol was the same in animals fed butter as in those fed coconut oil, and the triglycerides were lower in the latter group. Therefore, under our conditions, lauric and myristic acids cannot be highly hyperlipemic. From acute experiments with coconut oil in the dog,[24] it was concluded that saturated fatty acids of 12 and 14 carbon atoms had the most powerful effect in raising serum cholesterol, a finding that could also be applied to humans.[16]

However, the reason for this is far from clear, inasmuch as it has been reported that coconut oil decreases cholesterolemia in hyperlipemic patients[25] and that coconut oil fed to rabbits induced hypercholesterolemia only for 3 months,[26] an indication of the difficulties in drawing conclusions from short-term experiments. Under our conditions, the dietary feeding lasts for only 6 to 18 weeks, but in 6 to 10 weeks, owing to the type of diet, the animals are predisposed to the development of large thromboses that would normally occur in man, either in the coronary artery or in the veins,[27] only after 40 years of age. Therefore, in determining the value of our experimental model for reproducing human conditions, the results so obtained will have to be compared mainly with epidemiologic studies in man. As an example, it has been reported that the diet of the Atiu-Mitiaro islanders[28] is high in coconut. The serum cholesterol of the 40- to 49-year-old men is 235 mg per 100 ml, a value certainly no higher than those observed in man in North America, the Netherlands and Finland, where butter and dairy products certainly represent the largest portion of the dietary fat.[29]

It is known that olive oil has no cholesterol-lowering effect,[14] and in our experiments with oleic acid its cholesterolemic effects were not much different from those of stearic acid.[12, 13] In animals fed olive oil as the sole fat, the resulting cholesterolemia was slightly higher than with butter. Bovine tallow, which is much richer in oleic acid than butter, yielded a similar result. In all the experiments presented here, the lowest lipemia was recorded after feeding horse tallow (experiment 3), corn oil (experiment 2) and chicken tallow, horse tallow being richer in linolenic acid, and the two others in linoleic acid. In previous experiments,[12] these two acids were found to be the least thrombogenic and may have even antagonized the damaging effect of stearic and palmitic acids. The marked reduction in thrombogenicity that resulted from substitution of 6 per cent of the butter by corn oil in experiment 2 also suggest an antagonizing effect of linoleic acid.

With regard to thrombosis, our aim was to verify whether dietary fats with a high content in stearic and palmitic acids, the acids found to be the most thrombogenic in previous experiments,[12, 13] would be also the most thrombogenic. In experiment 2, cacao butter, by far the fat richest in stearic acid, was in fact the most thrombogenic; corn oil, the poorest in this fatty acid, was the least thrombogenic. These results with fats confirm those obtained with free fatty acids in previous[12, 13] and this series, in that only palmitic and stearic acids appeared to be highly thrombogenic. These data are also concordant with studies *in vitro* indicating that the long-chain saturated fatty acids are the most effective in aggregating blood platelets. In addition, in this experiment, with a few selected fats markedly different in the type of fatty acids they contained, almost all of the parameters examined (cholesterol, triglycerides, clotting time) appeared to be related to the severity of thrombosis.

The atherosclerosis experiment also confirms our earlier results that the most thrombogenic fatty acids are also the most atherogenic, independently of their

lipemic effect. Olive oil, which is more hyperlipemic than coconut oil, appears to be much less atherogenic and also much less thrombogenic. We had already found that oleic acid was almost no more thrombogenic[13] than linoleic acid, and only slightly more atherogenic.[12, 13] However, we also found that purified triolein was as thrombogenic as tristearin.[13] Under the present conditions, the only interpretation that can be offered for the surprisingly low thrombogenicity of olive oil and mostly bovine tallow is that oleic acid, whether in its free or in its triglyceride form, is not thrombogenic. Another surprising result is the thrombogenicity of arachis oil despite its high content in oleic and linoleic acid. This, however, confirms exactly what we had previously reported[11] and it is possible that, in this fat, the small quantity of long-chain fatty acids could be very active under the present conditions.

The most deceptive result is perhaps the fact that in experiment 2 a close relationship is obvious between various parameters and the severity of thrombosis, whereas in experiment 3, where a large number of various fats were used, none of the parameters examined could really be utilized for predicting the severity of thrombosis, a result similar to that already reported with various fatty acids.[12, 13] Of course, large thromboses cannot be initiated by endotoxins in animals with a serum cholesterol level below 200 mg and with a clotting time longer than 180 sec. On the basis of a large scale experiment, however, all that can be said is that these parameters represent no more than risk factors and by no means serve to predict closely the thrombotic tendency. From our experience with the addition of free fatty acids to the diet, these results are not surprising, because we have observed that the thrombogenicity of the different fatty acids does not necessarily parallel their effect on serum cholesterol or triglycerides. If, however, as suggested by these experiments, the thrombotic tendency is mostly due to the effect of some fatty acids on blood platelets, the parameters examined, except to some extent the clotting time, do not indicate this. Nevertheless, although the plasma clotting time as performed under our conditions is a highly constant and reliable determination, it is much more affected by the environmental conditions of the animal than the thrombotic phenomenon itself and, therefore, does not appear to constitute the ideal test.

Under the present conditions, it seems clear that neither the origin of a fat nor its degree of saturation governs its lipemic, thrombogenic or atherogenic effects. The type of fatty acids it contains is probably responsible for the above-mentioned effects; but due to the number of fatty acids involved and the various interactions observed to occur with such dietary feeding,[30] many more experiments will need to be done before we can completely predict the lipemic and thrombogenic effects of certain fats or fat mixtures. Also, with so many data to deal with, the help of a computer will probably be necessary, as other investigators have realized under similar conditions.[30] Another source of error is that, except for four samples, the composition in fatty acids of the fats used here has not been determined and could be slightly different from this reported in table 3.

As regards the mortality rate and the survival time, in almost none of the present or previous experiments did we find it to be related to the severity of the occlusive thrombosis. At least on a group basis, the survival time can be correlated with the degree of lipemia, as previously observed,[10] and the mortality is probably attributable to shock. In rabbits treated by injection with bacterial endotoxin, it was also reported that the mortality rate was related to the triglyceride level.[31]

From a practical point of view, it seems essential to evaluate to what extent the present experimental model in the rat yields results that are comparable to those obtained in man and also the degree to which it could help to clarify the pathogenesis of large thrombosis and coronary disease in man. Under our conditions, after cacao butter, which has also been found to be highly atherogenic in the rabbit,[20, 32] the most thrombogenic fat is butter and, therefore, probably the other dairy products also. Dairy products represent a large portion of the dietary fat of the highly civilized countries with a high rate of coronary disease. Coconut oil, although able to induce a degree of cholesterolemia similar to that of butter, does not seem to be highly thrombogenic in man[28] or under our experimental conditions. Olive oil, like oleic acid a nonthrombogenic fat, is largely used in the diet of countries such as Italy or Greece where the incidence of coronary disease is not as high as in northern Europe and North America.

These are only a few examples of the relationships that should be underlined and, it is hoped, that will stimulate fruitful collaboration between the experimental approach on the animal and epidemiologic studies in human.

SUMMARY

Among the more common saturated fatty acids, palmitic acid appears to be the most hyperlipemic when added to the basic high-fat diet of rats. Under these conditions, stearic and especially lauric acid seem to have much less effect on both the cholesterol and triglyceride serum levels. However, the rats fed the long-chain fatty acids, palmitic and mostly stearic acids, presented the most severe phlebothromboses, initiated in these rats by the intravenous injection of a *S. typhosa* endotoxin and resulting in multiple red hepatic infarcts.

Fats richer in stearic acid, such as cacao butter and butter, are also much more thrombogenic than lard and corn oil, which contain less stearic acid and much more linoleic acid, probably an antithrombogenic fatty acid.

Among a large number of fats fed to rats under comparable conditions, the results obtained were not as clear because butter was the most thrombogenic and olive oil and bovine tallow, despite a high content in stearic acid, the least thrombogenic. The low thrombogenicity of these latter fats, despite a marked hyperlipemic effect, is probably due to their high oleic acid content. However, the thrombogenicity of a fat is probably the result of multiple fatty acid interactions and should be further analyzed.

Only with a limited number of fats could the lipemia or the plasma

clotting time be utilized to predict the thrombotic tendency of the animals. But in large-scale experiments, serum cholesterol and triglycerides, as well as the plasma clotting time, could be considered merely as risk factors. In contrast to this, there was a highly significant correlation between the survival time of the endotoxin-treated animals and their serum triglyceride level.

The effects of a few selected fats on the production of aortic fatty streaks in the rat seem to be related to the thrombogenic effect and also to the composition in fatty acids. Under the conditions of this study, neither the degree of saturation of a fat nor its origin (animal, vegetable) appears to be of any use for predicting its lipemic, thrombogenic or atherogenic effects.

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Hepatic Metabolism of 1-^{14}C Octanoic and 1-^{14}C Palmitic Acids

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Abstract

The hepatic metabolism of 1-^{14}C octanoic acid was compared with that of 1-^{14}C palmitic acid in male rats which were fed. After intraportal injection only 1/6 to 1/15 as much octanoic acid as palmitic acid was incorporated into hepatic lipids. In contrast, octanoic acid yielded two to four times as much water-soluble product as did palmitic acid. Similar, but even more impressive, differences between the incorporation of these fatty acids into hepatic lipids were observed in liver slices incubated with ^{14}C octanoate and ^{14}C palmitate. The oxidation of octanoate to CO_2 was more than 10 times as great as that of palmitate. With both substrates, triglycerides comprised almost half the labeled lipid recovered. However octanoate yielded a higher proportion of labeled, unesterified fatty acids and a lower proportion of labeled phospholipid and monoglycerides than did palmitate. Most of the ^{14}C recovered in hepatic lipids after incubation with 1-^{14}C octanoate was found in the carboxyl groups of long-chain fatty acids, suggesting that the latter had been synthesized from 2-carbon fragments formed from the oxidation of octanoate. In contrast, only a small fraction of the palmitate was elongated.

The similarities and differences between the metabolism of octanoic and palmitic acid in liver and intestine, and the possible nutritional significance of octanoic acid are discussed.

Introduction

TRIGLYCERIDES containing medium-chain fatty acids and those containing long-chain fatty acids differ with respect to their intestinal absorption and intramucosal metabolism (1–6). Thus long-chain triglycerides (LCT) undergo intraluminal hydrolysis to monoglycerides, fatty acids, and glycerol by pancreatic lipase before absorption, are resterified to triglyceride within the mucosa, and are then exported as chylomicra via the lymphatics. In contrast, medium-chain triglycerides (MCT), containing principally octanoic and decanoic acids, can be absorbed intact, undergo hydrolysis within the mucosa by a mucosal lipase (4–6), and are then transported to the liver via the portal vein either as a salt or as an albumin complex (1–3).

Little attention has been given to a consideration of possible differences in the metabolism of medium- and long-chain fatty acids once they reach the liver. In the present report, evidence, based on both in vivo and in vitro experiments in the rat, is presented to show that medium-chain and long-chain fatty acids differ also with respect to their metabolism in the liver.

Methods

The male Sprague-Dawley type of albino rats (Charles River Rat Farm, North Wilmington, Mass.), weighing 180–250 g and maintained on Purina Lab Chow, were used in all experiments and were not fasted.

1-^{14}C octanoic acid and 1-^{14}C palmitic acid (New England Nuclear Corporation, Boston, Mass.) were purified by thin-layer chromatography prior to use. After dilution to the appropriate specific activity with unlabeled fatty acid (Hormel Foundation, Austin, Minn.) the mixture was dissolved in a small volume of diethyl ether and homogenized in a pH 7.4 Krebs-Ringer bicarbonate buffer (½ calcium), containing 10% Janel fatty acid-poor bovine albumin (Gallard-Schlesinger Chemical Manufacturing Corporation, Carle Place, New York), until the ether was evaporated.

Experiments in vivo

After anesthetization of rats with Nembutal the portal vein was isolated through a midline incision and injected in 1.0 min with 1.2 μMoles (2.4 μc) of either ^{14}C octanoic or ^{14}C palmitic acid in 1.0 ml of buffer-albumin solution. A 1-ml tuberculin syringe and 23-gauge needle were used for injection, and a pledget of Gelfoam was affixed to the injection site to ensure hemostasis.

Each of the fatty acids was given to a group of five rats. Livers were removed 1,3,5,10, and 15 min after completion of the injection and were then weighed and homogenized in a Waring Blendor with four volumes of Krebs-Ringer bicarbonate buffer. Lipids were extracted by the method of Folch et al. (7). In experiments with octanoic acid, the chloroform-methanol used for extraction contained, as carrier, 2.3 mM octanoic acid and 0.3 mM trioctanoin and was adjusted to pH < 3.0, as previously recommended (4). In experiments with either fatty acid substrate, radioactivity was determined after evaporating the lipid-containing phase to dryness in a rotary evaporator at 40C and redissolving the lipid in hexane. This method yielded recoveries of 88–92% in control albumin solutions containing either ^{14}C octanoic or ^{14}C palmitic acid. Radioactivity in water-soluble products was measured in a dioxane-counting solution (8). In all cases, counting efficiency was determined by a channels ratio method (9).

Experiments in vitro

Immediately after the sacrifice of the rats by decapitation, the liver was removed and sliced with a Stadie-Riggs tissue slicer. Liver slices, weighing 0.4 ± 0.02 g, were incubated in 5 ml of pH 7.4 Krebs-Ringer bicarbonate buffer (½ calcium), containing 5% albumin and 3 μMoles of ^{14}C fatty acid substrate. The buffer mixture was gassed with 5% CO_2–95% oxygen for 30 min prior to the introduction of the liver slices and again just prior to affixing the serum stoppers. Incubations were carried out in 25-ml Erlenmeyer flasks, fitted with center wells, in a Dubnoff shaker at 37C.

To measure the conversion of ^{14}C octanoic or ^{14}C palmitic acid to $^{14}CO_2$, the reaction was stopped by transferring the flask to an ice bath and adding 0.2 ml of 10 N H_2SO_4 to the main compartment and 0.5

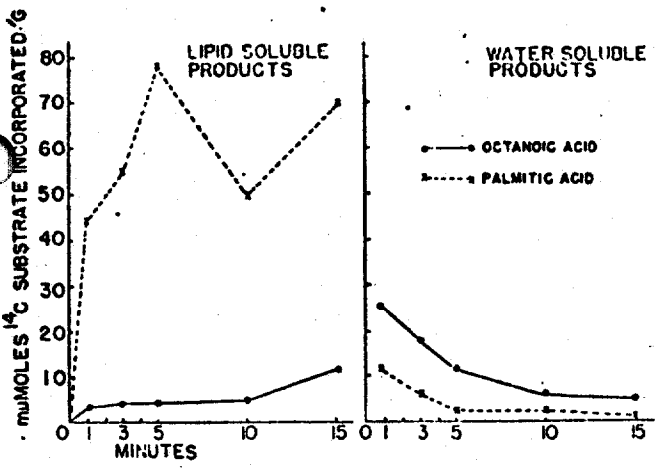

FIG. 1. Incorporation of 1-^{14}C octanoic acid and 1-^{14}C palmitic acid into lipid and water-soluble products of rat liver after intraportal injection.

Watertown, Mass.) to the center well. The flasks were shaken in ice for 30 min, after which the hyamine was transferred to 15 ml of 0.04% Liquifluor in toluene for an assay of radioactivity. Under these conditions $^{14}CO_2$ collection was quantitative, and less than 0.1% of the ^{14}C octanoate was volatilized and taken up by the hyamine. To measure conversion of the fatty acid substrate to lipid and water-soluble products, the reaction was stopped by transferring the flasks to an ice bath, after which the liver slices were removed, washed × 3 in ice-cold distilled water, homogenized with 2.0 ml of Krebs-Ringer bicarbonate buffer at 4C, extracted and assayed for radioactivity, as in the in vivo experiments. In addition, aliquots of the lipid-containing phase were subjected to a) thin-layer chromatography to separate their lipids into classes and to determine the radioactivity in each (10), and b) gas-liquid chromatography (GLC) to analyze the fatty acid composition and isotope content (11). The radioactivity of the carboxyl carbon of the fatty acids was determined, after saponification of the lipid extract, by a modification of the Schmidt reaction (12, 13).

Results

Experiments in vivo

As shown in Figure 1, far less octanoic acid than palmitic acid was incorporated into hepatic lipids

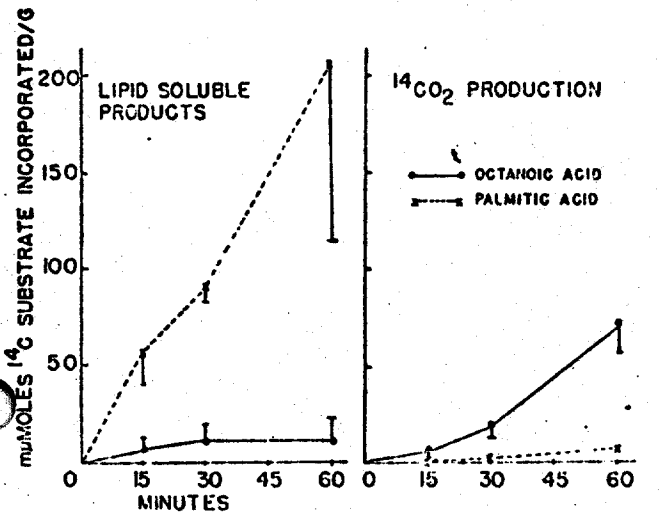

FIG. 2. Incorporation of 1-^{14}C octanoic and 1-^{14}C palmitic acid into lipid-soluble products and $^{14}CO_2$ by rat liver slices. Each point represents the mean ± 1 S.D. of three experiments.

TABLE I

Metabolism of 1-^{14}C Octanoic and 1-^{14}C Palmitic Acid to $^{14}CO_2$ and Lipid-Soluble Products by Liver Slices[1]

Substrate	$^{14}CO_2$	Lipid-soluble products
(3 μMoles)	(mμMoles ^{14}C fatty acid incorporated/g liver slice)	
Octanoic acid	212.7 ± 26.3	14.0 ± 4.3
Palmitic acid	15.8 ± 2.5	307.0 ± 15.0
p value	<0.01 (n = 3)	<0.001 (n = 6)

[1] Incubations were for 2 hr. Results expressed as mean ± SEM.

after the injection of these fatty acids into the portal vein. During the 15-min period of observation the ratio of octanoic:palmitic acid incorporation ranged between 1:6 and 1:18; the difference between the two was evident as early as 1 min after the injection. The amount of labeled lipid in the liver after injection of either fatty acid tended to increase with time, suggesting that, under the conditions of this experiment, repeated passages through the liver were required for extraction of the fatty acids from the plasma.

In contrast to its slower rate of incorporation into hepatic lipids, octanoic acid yielded two to four times more water-soluble material than palmitic acid. After injection of either fatty acid the concentration of labeled water-products in the liver was maximal at 1 min; then fell progressively (Figure 1).

Experiments in vitro

When liver slices were incubated with fatty acids, significantly less octanoic acid than palmitic acid was incorporated into hepatic lipids, as in the in vivo experiments (Figure 2). In contrast, the oxidation of octanoic acid to CO_2 by liver slices was significantly greater than that of palmitic acid. As shown in Table I, a high proportion of the labeled octanoic acid taken up by liver slices during a 2-hr incubation period was oxidized to CO_2, and very little was incorporated into lipid-soluble products. The converse was true of palmitic acid.

The lipid-soluble products formed from octanoic and palmitic acid in this experiment were characterized by separating the lipid classes by TLC (Table II). Triglycerides accounted for approximately half the radioactivity incorporated into the lipids of incubated liver slices. However the other lipid products of these two fatty acids differed in that octanoic acid yielded a higher proportion of unesterified fatty acids and a lower proportion of phospholipids and monoglycerides than did palmitic acid.

The lipid-soluble products were further characterized by GLC of the fatty acid methyl esters which were formed by transmethylation of the lipid extract. This revealed that fatty acids accounted for 92.8 ± 2.0% of the lipid radioactivity in the octanoate experiments and for 96.0 ± 0.4% in the palmitate experiment, indicating that little conversion of these

TABLE II

Lipid-Soluble Products Formed from 1-^{14}C Octanoic and 1-^{14}C Palmitic Acid by Liver Slices[1]

	Percentage distribution of total lipid radioactivity		
^{14}C Substrate (3 μMoles)	Phospholipids and monoglycerides	Unesterified fatty acids	Triglycerides
Octanoic acid (6)	19.3 ± 2.4	38.2 ± 6.9	48.3 ± 4.6
Palmitic acid (6)	34.7 ± 1.7	17.0 ± 1.8	46.3 ± 5.6
p value	<0.001	<0.05	NS

[1] Lipid-soluble products migrating with diglycerides, cholesterol, and cholesterol esters comprised 5.2% ± 1.2 of the lipid-soluble products from octanoic acid and 5.0% ± 0.3 from palmitic acid. Numbers in parenthesis refer to the number of experiments. The same six rat livers were used for these data as were employed for the lipid products data in Table I. Incubations were for 2 hr. Results expressed as mean ± SEM.

TABLE III

Distribution of ^{14}C Among Fatty Acids After Incubating Liver Slices with 1-^{14}C Octanoic or 1-^{14}C Palmitic acid[1]

Substrate (3 μMoles)	Fatty acid product					Fatty acid label in carboxyl groups
	<16:0[2]	16:0	16:1	18:0	18:1, 18:3 20:0, 20:4	
	(% Total fatty acid label in each)					(%)
Octanoic acid	17.5 ± 6.4	42.2 ± 8.6	12.5 ± 2.2	4.8 ± 0.5	23.0 ± 5.1	80.1 ± 2.4
Palmitic acid	1.8 ± 0.8	77.4 ± 2.9	12.0 ± 1.7	4.0 ± 0.8	4.8 ± 0.7	96.8 ± 1.1
p value	<0.05	<0.01	NS	NS	<0.05	<0.001

[1] The same six rat livers were used for these data as were employed for the data in Table II. Incubations were for 2 hr. Results expressed as mean ± SEM.

[2] <16:0 refers to fatty acids the carbon chain-lengths of which are less than 16 but greater than six.

substrates to glyceride glycerol, phospholipid bases, or cholesterol had occurred. From the data in Table III it can be seen that, after 2 hr of incubation, less than 10% of the label in ^{14}C palmitic acid was found in hepatic fatty acids of longer chain-length. In contrast, most of the ^{14}C octanoic acid underwent elongation. Of particular note, less than 2% of the fatty acid radioactivity after incubation was recovered in the octanoate fraction. Moreover a large proportion of the label (23%) was found in C18 and C20 unsaturated fatty acids. Since 80% of the ^{14}C in these longer-chain fatty acids which were derived from 1-^{14}C octanoic acid was present in carboxyl groups (Table III), it is highly probable that the octanoate was oxidized to acetate prior to its incorporation into long-chain fatty acids.

Discussion

From the data presented it is evident that, compared with palmitic acid, octanoic acid is a poor substrate for lipid synthesis in the liver but is more readily catabolized to CO_2 and water-soluble products. Similar differences in the metabolism of these two fatty acids have been observed in intestinal slices (5). However their behavior in the liver differs from that in the intestine in several respects. Whereas, in the liver, the bulk of the octanoic acid that is not catabolized to CO_2 and water-soluble products is converted to long-chain fatty acids, little of the octanoic acid that enters the intestinal mucosa is elongated; most of the uncatabolized fraction was recoverable intact and unesterified. Elongation of palmitic acid occurs to a small but significant degree in the liver but almost not at all in the intestinal mucosa. In the liver, comparable fractions of fatty acids derived from octanoic and palmitic acid are incorporated into triglycerides whereas, in the intestine, there is preferential incorporation of fatty acids derived from palmitic acid but not octanoic acid.

Previous studies have shown that excluding the liver from the circulation does not significantly reduce the amount of $^{14}CO_2$ recovered in the expired air of animals given 1-^{14}C octanoic acid (20). This finding suggests that octanoic acid may be preferentially oxidized to CO_2 rather than incorporated into other lipids not only in the liver and intestine but also in other extrahepatic tissues. It is quite possible therefore that octanoic acid, in supporting growth in man (15) and animals (16,17), merely spares carbohydrate and fat by providing calorie energy.

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5. HEPATIC METABOLISM OF MEDIUM CHAIN FATTY ACIDS

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TRIGLYCERIDES CONTAINING MEDIUM CHAIN LENGTH FATTY ACIDS differ markedly from those containing long chain fatty acids with respect to their intestinal absorption and intramucosal metabolism, as has been discussed. Long chain triglycerides undergo intraluminal hydrolysis to monoglycerides, fatty acids, and glycerol, are absorbed, and these products are recombined within the intestinal mucosa to form triglycerides, and are then exported as chylomicra via the lymphatics. In contrast, medium chain triglycerides, which contain principally octanoic acid, can be absorbed without prior hydrolysis although intramucosal lipolysis occurs. Medium chain fatty acids released by the action of an intramucosal lipase are transported to the liver via the portal vein, as shown by Bloom, Chaikoff and Reinhardt (1951), Kiyasu, Bloom, and Chaikoff (1952), and by Borgström (1955).

The possibility that medium chain and long chain triglycerides also differ with respect to their metabolism in other tissues has not been examined previously. Since medium chain triglycerides are being used therapeutically, with apparent good effect, in the management of steatorrhea due to a variety of causes, the metabolic fate of such lipid is of considerable interest and possible importance. The data to be presented, based on *in vitro* and *in vivo* studies in the rat, show that in all tissues examined, including liver, kidney, heart, striated muscle, brain and adipose tissue, octanoic acid is much more readily oxidized to CO_2, but less effectively incorporated into tissue lipids, than palmitic acid.

In vivo studies were performed by injecting into the portal vein either octanoate-C^{14} or palmitate-C^{14} as an albumin complex, extracting the hepatic lipid and measuring its radioactivity. As can be seen in Fig. 5-1,

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LIPID FORMATION IN VIVO FROM MEDIUM & LONG CHAIN FATTY ACIDS BY RAT LIVER

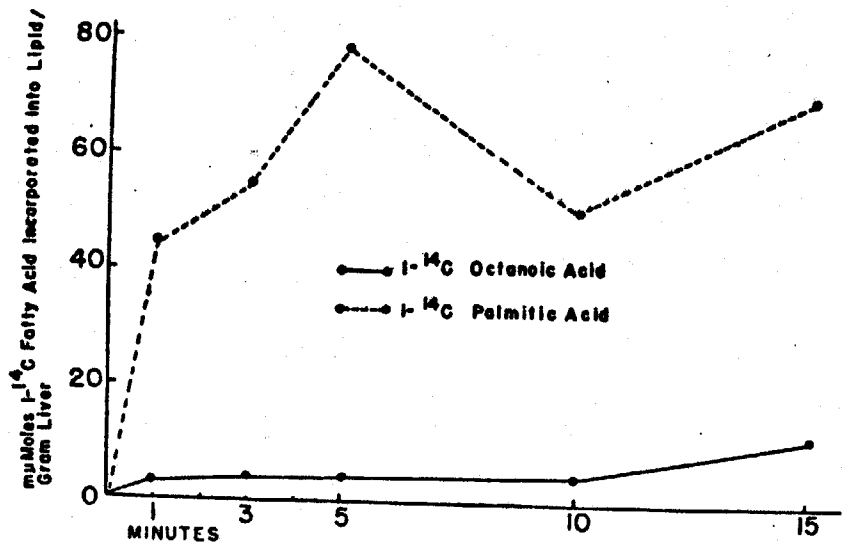

Fig. 5-1. Male rats weighing 180-220 g were anesthetized with Nembutal (pentobarbital sodium) and the portal vein was isolated through a midline incision. One ml of a solution containing 1.2 μ moles (2.4 μc) of either octanoate-1-C14 or palmitate-1-C14 complexed by homogenization with 10% Janel fatty acid-poor bovine albumin in pH 7.4 Krebs-Ringer bicarbonate buffer was injected into the portal vein in 1.0 minute with a 1 ml tuberculin syringe and a 23-gauge needle. The livers were removed 1,3,5,10, and 15 minutes after injection, weighed, and homogenized in a Waring Blender with 4 volumes of Krebs-Ringer bicarbonate buffer. The lipids were extracted with chloroform–methanol (Folch et al., 1957), modified for medium chain fatty acid recovery (Playoust and Isselbacher, 1964). Isotope content was determined by liquid scintillation spectrometry after evaporating the lipid-containing phase to dryness in a rotary evaporator at 40 C, redissolving the lipid in hexane, and transferring an aliquot to a 0.04% solution of Liquifluor in toluene.

far less octanoic acid than palmitic acid was incorporated into hepatic lipid. The difference was apparent as early as one minute following injection and persisted over the 15-minute period of observation. At no time did the amount of octanoate incorporated into lipid soluble products exceed one-sixth that of palmitate.

As is evident from Fig. 5-2, the results obtained from *in vitro* studies confirmed the *in vivo* observations. Liver slices were prepared from 180-220 g male rats and incubated with the carboxyl-labeled fatty acid in a buffer containing albumin. Again, the rate of incorporation of octanoic acid into lipid soluble products was much less than that of palmitic acid. In contrast to its slower rate of incorporation into hepatic lipids, octanoate was much more readily oxidized to carbon dioxide than was palmitic acid.

LIPID & $^{14}CO_2$ FORMATION FROM MEDIUM & LONG CHAIN FATTY ACIDS BY RAT LIVER SLICES

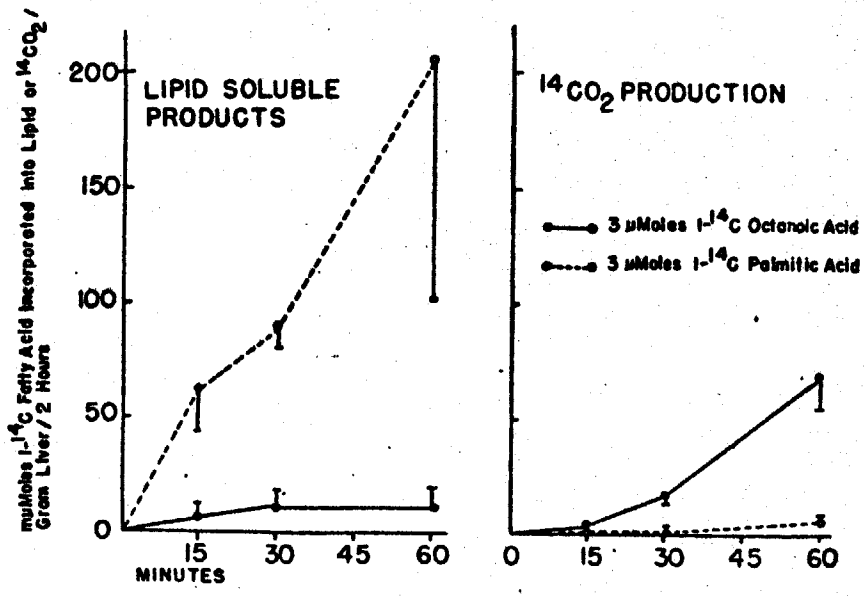

Fig. 5-2. Liver slices prepared with a Stadie-Riggs tissue slicer and weighing 0.4± 0.02 g were incubated in 5 ml of Krebs-Ringer bicarbonate (½ calcium) containing 5% albumin and 3 μ moles of the C14 fatty acid substrate. The buffer mixture had been gassed with 5% CO_2/95% O_2 prior to the introduction of the slices and again before stoppering with serum caps. The incubations were in a Dubnoff shaker at 37 C in 25-ml Erlenmeyer flasks fitted with center wells. $C^{14}O_2$ production was determined by liquid scintillation spectrometry after transferring flasks to an ice bath, adding 0.2 ml H_2SO_4 to the main compartment, 0.5 ml Hyamine hydroxide to the center well, shaking in ice for 30 minutes and transferring the Hyamine to 15 ml of 0.04% Liquifluor in toluene. Lipid-soluble product formation was determined in separate flasks as in the *in vivo* experiments described in Fig. 5-1 after the slices had been removed, washed three times in ice cold distilled water, homogenized with 2.0 ml Krebs-Ringer bicarbonate buffer at 4 C, and the lipids extracted. Each point represents the mean ± 1 SD of three experiments.

Because the previous nutritional state of the animal is known to modify lipid metabolism (Lossow and Chaikoff, 1955), the oxidation of medium and long chain fatty acids and their incorporation into lipid were measured in liver slices from male rats either fed or fasted for 17 hours prior to sacrifice. As can be seen from Table I, fasting depressed both the oxidation of octanoic acid to $C^{14}O_2$ and its incorporation into hepatic lipid. Nevertheless, the amount of octanoic acid oxidized was still substantial. In contrast, fasting decreased the oxidation of palmitate to a greater extent than octanoate but had no effect on the incorporation of palmitate into hepatic lipid.

The data in Table II show that the sex of the rat from which the liver was derived did not alter either the oxidation of octanoic acid or its incorporation into lipid.

Table I

Metabolism of Medium and Long Chain Fatty Acids to CO_2 and Lipid Soluble Products by Rat Liver Slices[a]

I. EFFECT OF PREVIOUS DIET

Dietary Regimen	Substrate (3 μmoles)	$C^{14}O_2$ Production	Lipid soluble products
		mμmoles fatty acid-1-C^{14} incorporated per gram liver per 2 hours	
Fed	Octanoic acid-1-C^{14}	255 ± 87	8 ± 2
Fasted	Octanoic acid-1-C^{14}	127 ± 20	2 ± 0
		$P<0.001$	$P<0.001$
Fed	Palmitic acid-1-C^{14}	26 ± 7	232 ± 18
Fasted	Palmitic acid-1-C^{14}	7 ± 1	239 ± 14
		$P<0.01$	NS[b]

[a] Methodology was identical to that used in the experiments in Fig. 5-2 except that all incubations were for 2 hours. Liver slices were prepared from 7-week-old male rats weighing on the average 210 g which had been either fed or fasted 17 hours before sacrifice. Each number represents the mean ±1 SD of six animals and each liver provided slices to assay either $C^{14}O_2$ or lipid production from both fatty acid substrates. The statistical significance of differences between groups was calculated by the paired "t" test (Mainland, 1963).
[b] NS = not significant.

Table II

Metabolism of Medium and Long Chain Fatty Acids to CO_2 and Lipid Soluble Products by Rat Liver Slices[a]

II. EFFECT OF SEX OF DONOR

Sex	Substrate (3 μmoles)	$C^{14}O_2$ Production	Lipid soluble products
		mμmoles fatty acid-1-C^{14} incorporated per gram liver per 2 hours	
Male	Octanoic acid-1-C^{14}	85 ± 46	5 ± 2
Female	Octanoic acid-1-C^{14}	104 ± 44	6 ± 3
		NS[b]	NS
Male	Palmitic acid-1-C^{14}	8 ± 4	247 ± 2
Female	Palmitic acid-1-C^{14}	10 ± 2	304 ± 27
		NS	$P<0.05$

[a] Methodology was identical to that used for the data in Table I. All rats were fed until sacrifice. They were 7 weeks old with an average weight of 215 g for the males and 195 g for the females.
[b] NS = not significant.

While the age of the animal apparently did not influence the metabolism of palmitate by the liver, there seemed to be some decrease in the oxidation of octanoate with advancing age (Table III).

Table III

Metabolism of Medium and Long Chain Fatty Acids to CO_2 and Lipid Soluble Products by Rat Liver Slices[a]

III. EFFECT OF AGE OF DONOR

Age (weeks)	Substrate (3 μmoles)	$C^{14}O_2$ Production	Lipid soluble products
		mμmoles fatty acid-1-C^{14} incorporated per gram liver per 2 hours	
4½	Octanoic acid-1-C^{14}	173 ± 40	3 ± 3
11	Octanoic acid-1-C^{14}	117 ± 37	2 ± 2
		$P<0.05$	NS[b]
4½	Palmitic acid-1-C^{14}	11 ± 3	222 ± 30
11	Palmitic acid-1-C^{14}	8 ± 4	204 ± 27
		NS	NS

[a] Methodology as in Table I. Male rats fed until sacrifice were the donors of the liver slices and the average weight of the 4½-week-old rats was 110 g and of the 11-week-old rats 335 g.
[b] NS = not significant.

It might be noted that in all the preceding experiments standard deviations were rather wide, indicating either variability in preparing tissue slices or more probably, variability from animal to animal. Nevertheless, the differences in the metabolism of octanoic and of palmitic acid were quite consistent and highly significant under all experimental conditions.

In order further to explore possible differences between the metabolism of medium and long chain fatty acids, their lipid products in the livers were characterized (Table IV). The lipid classes were separated by thin layer

Table IV

Lipid Soluble Products Formed from Medium and Long Chain Fatty Acids by Rat Liver

Substrate (3 μmoles)	Per cent distribution of total lipid radioactivity		
	Triglycerides	Unesterified fatty acids	Phospholipids and monoglycerides
Octanoic acid-1-C^{14}	48 ± 5	38 ± 7	10 ± 2
Palmitic acid-1-C^{14}	46 ± 6	17 ± 2	32 ± 2
	NS[b]	$P<0.05$	$P<0.001$

[a] Liver slices from six fed male rats (average weight 200 g) were incubated for 2 hours and the lipids were extracted. The methods used are described in Table I. Of the octanoic acid 14.0 ± 4.3 and of the palmitic acid 307.0 ± 15.0 mμmoles was incorporated into lipid per gram liver ($P<0.001$). Aliquots of the lipid extract were subjected to thin layer chromatography to separate the lipids into classes and to determine the radioactivity of each.
[b] NS = Not significant.

chromatography and their isotope content determined (Scheig and Isselbacher, 1965). With both C^{14} fatty acids, approximately half the radioactivity recovered in lipid was found in triglyceride. However, the other lipid products of these two fatty acids differed significantly. Thus, 38% of the lipid radioactivity from octanoate but only 17% of that from palmitate was in the form of unesterified fatty acids. In contrast, only 10% of the octanoic acid but 32% of the palmitate was incorporated into monoglycerides and phospholipids.

The fatty acid constituents of these lipid products were determined by preparing the fatty acid methyl esters and assaying the isotope content of each after gas-liquid chromatographic separation (Scheig, Alexander, and Klatskin, 1966). Interestingly, when octanoic and palmitic acids served as substrates, 93% and 96%, respectively, of the lipid radioactivity was recovered in the fatty acid moieties of the complex lipids, indicating that neither substrate had been converted to glyceride glycerol, phospholipid bases or cholesterol to any appreciable extent. As is evident from Table V,

Table V

Distribution of C^{14} amongst Fatty Acids after Incubating Liver Slices with Octanoate-1-C^{14} or Palmitate-1-C^{14}[a]

Substrate (3 μmoles)	(Per cent total fatty acid label in each)				
	<16.0[b]	16:0	16:1	18:0	18: and 20: unsaturated
Octanoic acid-1-C^{14}	17 ± 6	42 ± 9	13 ± 2	5 ± 1	23 ± 5
Palmitic acid-1-C^{14}	2 ± 1 $P<0.05$	77 ± 3 $P<0.01$	12 ± 2 NS[c]	4 ± 1 NS	5 ± 1 $P<0.05$

[a] The same six rat livers and the same methods were used for this data as were employed for the data in Table IV. Aliquots of the lipid extract were subjected to gas-liquid chromatography to analyze fatty acid composition and isotope content.
[b] <16:0 refers to fatty acids whose carbon chain lengths are less than 16 but greater than 6.
[c] NS = Not significant.

most of the palmitate was incorporated into tissue lipid unaltered, only 9% having been elongated and 12% desaturated. In contrast, the bulk of the octanoate incorporated into liver lipids was in elongated fatty acids, only 17% not having been converted to long chain fatty acids. Actually, less than 2% of the recovered lipid radioactivity remained in the form of octanoate. Moreover, a rather large proportion of the octanoate, namely 36%, was in fatty acids which were both elongated and desaturated, and the radioactivity was contained in 16, 18, and 20-carbon unsaturated fatty acids. Octanoate probably had been oxidized to acetate or acetyl coenzyme

A prior to being incorporated into long chain fatty acids either by synthesis via the malonyl-CoA pathway or, more likely, by 2-carbon chain addition to endogenous fatty acids in mitochondria (Harlan and Wakil, 1962; 1963). In support of the latter hypothesis, it was found by Schmidt degradation that 80% of the label in the fatty acids derived from octanoate was contained in carboxyl groups (Phares, 1951; Brady, Bradley, and Trams, 1962).

That medium chain fatty acids are more readily oxidized than long chain fatty acids was evident not only in liver and in the intestine (Greenberger, Franks, and Isselbacher, 1965), but also in other tissues. Thus, as shown in Fig. 5-3, the oxidation of octanoate was significantly greater than

OXIDATION OF MEDIUM & OF LONG CHAIN FATTY ACIDS

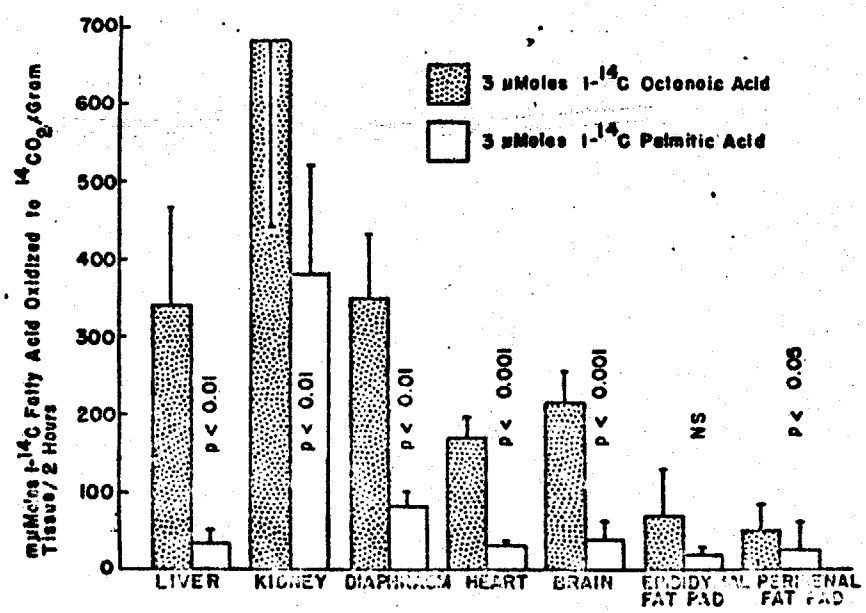

Fig. 5-3. Methodology was the same as that used for the data in Table I. Male rats, weighing on the average 250 g, were fed until sacrifice. One animal provided all the tissues. Each bar represents mean ± 1 SD of determinations on six animals. The average time from sacrifice until start of incubations was 40 minutes. The slices in each flask weighed about 100 mg except for brain which averaged 50 mg, and adipose tissue which averaged 150 mg.

palmitate in kidney, diaphragm, heart, brain, and adipose tissue. Noteworthy is the magnitude of the oxidation observed in all these issues. In contrast to its extensive oxidation, the data in Fig. 5-4 sh[illegible] that little of

LIPID SYNTHESIS FROM MEDIUM & LONG CHAIN FATTY ACIDS

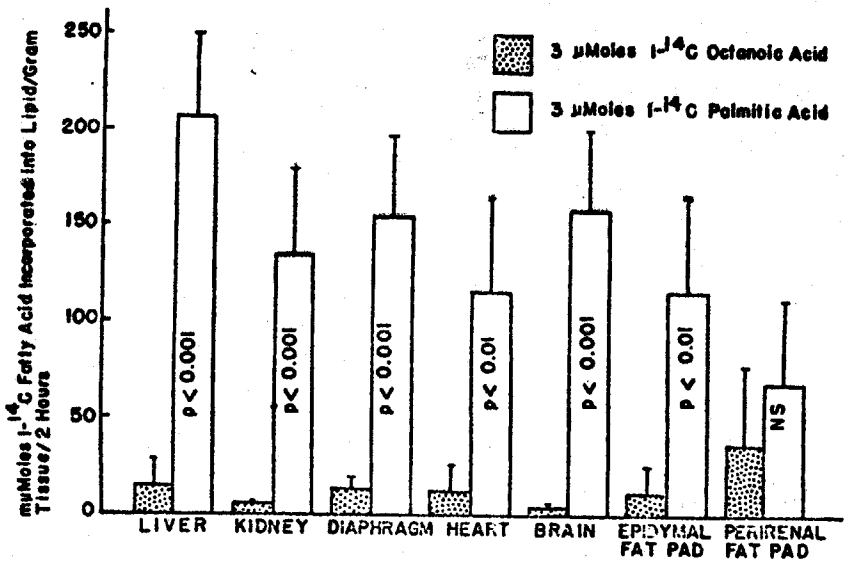

Fig. 5-4. See Fig. 5-3 and Table I methodology, text for discussion.

the octanoate was used for tissue lipid synthesis, especially when compared to tissue utilization of palmitate.

From the data presented, it is evident that, compared to long chain fatty acids, medium chain fatty acids are poorly utilized for tissue lipid synthesis but are readily catabolized to CO_2. In comparing our data on the liver with those of Greenberger, Franks and Isselbacher (1965) on the intestine, it is apparent that the type of lipid formed from octanoic acid differs in these two tissues. Thus, in the liver the bulk of the octanoic acid that is not catabolized is converted to long chain fatty acids which are esterified, while in the intestine little is used for elongation or esterification.

In summary, the metabolism of a representative medium and a long chain fatty acid was studied in the rat. Octanoic acid proved to be a relatively poor substrate for lipid synthesis compared with palmitic acid, but was more readily oxidized to CO_2. These data suggest that triglycerides containing octanoic acid may support growth by providing calories, thereby sparing carbohydrate and other fats from catabolism. Accordingly, the therapeutic use of medium chain triglycerides as a more readily absorbed dietary lipid in patients with malabsorption due to biliary tract, pancreatic, or intestinal disease would appear to have a rational basis.

Acknowledgments

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Questions

Chairman Senior: From your data, where you analyzed the liver fatty acids after octanoate metabolism, you indicated that a fairly large fraction of the recovered radioactivity was in longer fatty acids, indicating elongation. Is the mechanism of this elongation a preliminary breakdown to acetate and then a resynthesis of fatty acids *de novo* (in which case the label would be distributed throughout the chain), a mitochondrial addition of labeled acetate to fatty acids which would place the label in the C-1 position, or is it a direct elongation of the octanoate by other 2-carbon additions? Do you have any evidence for direct elongation of the octanoate, in which case the C-1 label would be found in C-9 or C-11 position?

Dr. Scheig: As you know, fatty acid synthesis can occur either *de novo* from 2-carbon fragments by the malonyl-CoA pathway in the cell sap or by an elongation reaction, in which a 2-carbon fragment is added to a preexisting fatty acid. This latter occurs principally in the mitochondria and perhaps somewhat in the microsomes. There are two lines of evidence that help me to distinguish which synthetic process is being used during octanoate metabolism: First, when the fatty acids recovered after the two-hour incubation were decarboxylated, that is the terminal CO_2 group was removed and counted, it was found by Schmidt degradation that 80% of the fatty acid radioactivity was in that carboxyl group (Phares, 1951; Brady, Bradley, and Trams, 1962); second, when the fatty acid products which result from incubation of a liver slice with acetate-1-C^{14} or following the injection of acetate-1-C^{14} into the portal vein were studied, we found that the fatty acid products were quite similar to those observed when octanoic acid was the substrate. So I think both these sets of findings suggest that the octanoic acid had been oxidized to 2-carbon fragments and then added to a preexisting fatty acid, that is, the octanoic acid carbon is used in an elongation reaction.

Chairman Senior: Your opinion, then, is that the octanoate is catabolized to acetate first, almost exclusively, rather than being itself elongated.

Dr. Scheig: Right.

Dr. Hans Kaunitz (Columbia University, New York): I wanted to ask whether you noted that the linoleate content of the diet that you provided for your animals had any bearing on your results, because we have found repeatedly that the amount of linoleate in the diet has a strong bearing on the incorporation of medium chain triglycerides into tissue triglycerides. We found that a linoleate-free diet leads to no incorporation, but if you

increase linoleate in the diet up to 10–15%, the fatty acids from medium chain triglycerides will be incorporated into the liver triglycerides or tissue triglycerides.

Dr. Scheig: The diet used was Purina lab chow, which is 3.8% lipid, of which about a third is linoleate (Scheig, Alexander and Klatskin, 1966). I don't know how many grams of chow a day these animals were eating, so I couldn't calculate how much linoleate they were actually getting.

Dr. G. Steiner: In your studies comparing the metabolism of octanoate relative to palmitate you have shown decreased recovery of label in lipid and increased CO_2 production. This raises the possibility of increased lipid turnover and hence decreased recovery of labeled lipid because it has just been oxidized to CO_2. I would be interested to know what controls you have done to exclude this possibility.

Dr. Scheig: I'm not sure what kind of controls you would use to exclude it.

Dr. Steiner: Have you any data on the specific activity of the label in the lipids recovered? Have you any kinetic data on what is happening in this situation?

Dr. Scheig: No.

Dr. Peter R. Holt: Dr. Senior's comment about Robert Frost at the beginning of the symposium might well apply to medium chain fatty acids with respect to the diversion between esterifying pathway and oxidation. You have mentioned a number of tissues in your presentation. One tissue apparently differs and that is the mammary tissue, in which there is significant esterification of octanoate. It is intriguing, I think, and important in this type of symposium to speculate upon the reasons why these differences occur. Are these due only to enzyme specificities or could there be other mechanisms which will divert this particular type of lipid into these pathways?

Another point I would like to make is the possibility of the interrelationship of esterification with glucose metabolism. We have found in studies on intestinal mucosa that if you starve the animal until glycogen disappears and then attempt to show esterification with long chain lipid, it in fact decreases, while if in your incubation medium you add glucose, it increases. The intriguing point is that when one adds MCT, instead of glucose, one would still be able to show an increased esterification. This in fact is partly due to glycerol. Would you comment on that, too?

Dr. Scheig: I really have nothing to say on the latter point. On the former point we have no data. I think you could speculate that this might be due to different enzyme specificity. One could also speculate, as Dr. Hofmann mentioned earlier, that this had to do with different solubilities of the substrate. In order to compare these as closely as possible, both octanoate and palmitate were complexed with albumin. But perhaps the release of the octanoate from the albumin complex is more readily accomplished than is the release of palmitate. Tissue slices were used, and so the transport across the cell membrane still had to take place before either oxidation or incorporation into various lipid products could occur. I think there is a multiplicity of factors and we have no data to determine which of these factors are involved.

Dr. Daniel Rudman: I wanted to bring out a question you just touched on, Dr. Scheig. Spector and Steinberg (1965), in studying the utilization of fatty acids by tumor cells, showed that the primary determinant was the concentration of unbound fatty acid in the medium rather than the total quantity present. Undoubtedly with equivalent amounts of albumin and equal quantities of octanoate and palmitate your concentration of free octanoate must have been much greater than the concentration of free palmitate, so that before one could accept the concept that the medium chain fatty acid is much more readily utilized one would want to study the problem of equivalent concentrations of unbound fatty acid.

J. Appl Physiol. 19 (2): 335-337 (1964)

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Octanoic acid absorption and oxidation in humans[1]

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SCHWABE, ARTHUR D., LESLIE R. BENNETT, AND LANCE P. BOWMAN. *Octanoic acid absorption and oxidation in humans.* J. Appl. Physiol. 19(2): 335-337. 1964.—The absorption and oxidation of carboxyl-labeled octanoic acid as determined by monitoring of expired $C^{14}O_2$ was studied in 27 subjects. In 12 normal volunteers there was no significant difference in the recovery of $C^{14}O_2$ after oral and intravenous administration of uniformly labeled octanoic acid. Administration of 100 g sucrose by mouth did not seem to inhibit octanoic acid oxidation in ten subjects. Infusion of 1 g unlabeled octanoate also did not significantly affect the oxidation of radioactive octanoate in five subjects. These observations in humans tend to confirm animal studies which have shown that octanoic acid is readily absorbed from the gastrointestinal tract and rapidly oxidized. The oxidation of small amounts of this fatty acid appears to proceed at the same rate despite moderate blood levels of glucose or octanoic acid. These characteristics of short chain fatty acid absorption and oxidation can be exploited in the study of fat absorption in human subjects.

intestinal absorption fatty acid metabolism

OCTANOIC ACID, an 8-carbon fatty acid found in esterified form in coconuts, butter, and certain other dietary fats, has not been extensively used in clinical medicine. Animal studies have shown that octanoic acid is rapidly absorbed from the intestine, carried to the liver via the portal venous system, and rapidly and completely oxidized (6). It has also been observed in rats that the oxidation of carboxyl-labeled octanoic acid is relatively uninhibited by the administration of carbohydrates (7). These physiological properties of octanoic acid first suggested its use and that of its ester, glyceryl trioctanoate, in the study of intestinal absorption in man (8). The present study was undertaken to determine the degree of octanoate absorption and its oxidation under various conditions in human subjects.

METHODS

The water-soluble octanoic acid labeled with C^{14} in the carboxyl position, henceforth referred to as RAO (radioactive octanoate) for brevity, is prepared by the New England Nuclear Corporation with a specific activity of 1.99 mc/mmole. The isotope was administered in 2- to 3-μc doses in the fasting state to a group of normal volunteers and to patients without evidence of pulmonary, liver, or heart disease, or abnormal lipid metabolism. On every occasion following the administration of the isotope, each subject was immediately placed under a plastic helmet and the expired $C^{14}O_2$ was continuously monitored and recorded by a method outlined in a previous paper (8) (Fig. 1). The cumulative expired radioactive CO_2 recovered in a 50-min period was expressed as the percentage of the administered dose. The collection of expired $C^{14}O_2$ was terminated after 50 min because these and previous experiments have shown that peak recovery of $C^{14}O_2$ occurred invariably between 15 and 30 min. Thereafter $C^{14}O_2$ recovery was falling off steadily. Lossow and Chaikoff (7) have shown that the $C^{14}O_2$ recoveries in fed and fasted rats after 6 hr were 82 and 90%, respectively, following intravenous injection of octanoic acid. The hourly recovery of $C^{14}O_2$ was greatest during the first hour of testing.

The subjects were divided into three experimental groups, all of which received one or more intravenous injections of RAO to establish control recovery values of $C^{14}O_2$.

The first experiment, involving a group of 12 healthy medical students, was designed to compare the recovery of $C^{14}O_2$ after orally and intravenously administered RAO. Each subject received 2 or 3 μc of the labeled compound orally and intravenously on separate occasions at least 3 days apart. Monitoring of expired $C^{14}O_2$ was performed after each test dose (Table 1).

The second study performed on ten subjects (four normal adults and six patients) was designed to determine the effect of

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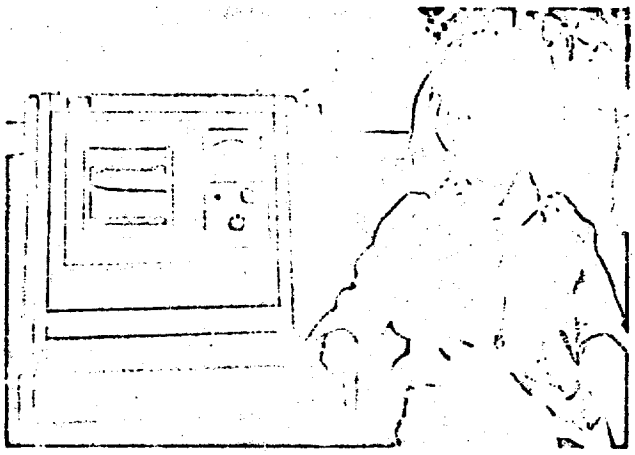

FIG. 1. Apparatus for measurement of expired radioactive carbon dioxide.

glucose administration on octanoic acid oxidation. Initially each of the ten subjects received one or more intravenous control injections of 2 μc RAO at least 3 days apart. Subsequently, again at least 3 days following the last test, each one received 100 g glucose dissolved in 250 ml lemon-flavored tap water. Twenty minutes after the glucose had been ingested each individual was placed under the helmet and given 2 μc of RAO intravenously. Monitoring of expired $C^{14}O_2$ was carried out after each test dose (Table 2).

In the third group of five patients the effect of fatty acid load on RAO oxidation was investigated. Three or more days after the base-line determination, each subject received an intravenous infusion of 1 g unlabeled octanoic acid dissolved in 250 ml 1.3% sodium bicarbonate at the rate of 60 drops/min. At the end of the infusion 2 μc RAO were again administered intravenously. The amounts of $C^{14}O_2$ recovered after each test dose are compared on Table 3.

TABLE 1. *Comparison of $C^{14}O_2$ recovery after oral and intravenous RAO administration in twelve normal males*

Subj.	A. Intravenous RAO			B. Oral RAO			A − B % Diff.
	Dose, μc	DT, min	% Recovered	Dose, μc	DT, min	% Recovered	
JM	2	2	16.5	2	4	15.7	+0.8
LM	2	1	14.9	2	4	17.7	−2.8
JH	2	2	15.1	2	3	16.3	−1.2
DY	3	2	15.2	3	3	16.1	−0.9
HB	3	2	17.5	3	3	18.1	−0.6
RP	3	1	16.7	3	4	16.5	+0.2
PA	2	1	12.7	2	3	16.3	−3.6
PM	2	2	15.3	2	4	14.8	+0.5
BD	2	2	18.2	2	5	13.9	+4.3
RA	2	2	19.0	2	5	13.0	+6.0
LB	2	2	12.0	3	6	11.0	+1.0
MW	3	2	15.1	3	4	15.5	−0.4
Avg.			15.7			15.4	

The average significance of comparison = $P > 0.7$.

RESULTS

In the first group of 12 medical students no statistically significant difference in the recovery of $C^{14}O_2$ after intravenous (column A) and oral (column B) administration of RAO was noted (Table 1). The delay time (DT), i.e., the interval from the point at which RAO was administered to the point at which activity was first observed on the recorder chart, ranged from 1 to 2 min for the intravenously administered and from 3 to 6 min for the orally administered isotope.

In the second group, 18 control and 10 postglucose intravenous RAO tests were performed in the ten subjects. No statistically significant differences of recovered $C^{14}O_2$ before and after glucose administration were noted, suggesting that glucose ingestion did not inhibit octanoic acid oxidation (Table 2).

The results of five control (column A) and five postoctanoic

TABLE 2. *Effect of glucose on RAO oxidation*

Subj.	A. Intravenous RAO Control			B. Intravenous RAO, After 100 g Glucose			A − B % Diff.
	Dose, μc	DT, min	% Dose recovered	Dose, μc	DT, min	% Dose recovered	
NB	2	2	21.9	2	2	24.5	−2.6
BR	2	2	24.0	2	2	31.4	−7.4
AS	2	3	24.5	2	1	24.9	−.4
JW	2	2	25.9	2	1	21.0	+4.9
MB	2	2	14.3	2	2	21.7	−7.5
	2	2	14.1				
SH	2	2	16.9	2	2	15.7	+.7
	2	2	15.9				
DP	2	2	20.0	2	1	20.5	+.3
	2	2	21.6				
BJ	2	2	18.1	2	2	21.0	−2.1
	2	1	18.3				
	2	1	20.4				
AM	2	3	17.2	2	2	20.2	−3.9
	2	2	14.5				
	2	2	17.4				
LA	2	1	19.0	2	2	19.7	−2.3
	2	1	15.7				
Avg.			18.9			22.0	

The average significance of comparison = $P > 0.2$.

TABLE 3. *Effect of unlabeled octanoic acid infusion on RAO oxidation*

Subj.	A. Control i.v. RAO			B. Postinfusion i.v. RAO				A − B % Diff.
	Dose, μc	DT, min	% Dose recovered	Octanoic acid load, g	Dose, μc	DT, min	% Dose recovered	
MP	2	2	19.3	1	2	3	18.8	+0.5
RA	2	2	19.4	1	2	2	16.2	+3.2
ET	2	2	19.6	1	2	3	20.3	−0.7
KB	2	2	22.8	1	2	3	24.2	−1.4
AH	2	2	22.4	1	2	3	21.5	+0.9
Avg.			20.7				20.2	

The average significance of comparison = $P > 0.7$.

infusion (column B) tests in the third group are compared in Table [illegible]. There was no statistically significant difference in the amount of $C^{14}O_2$ recovered in these determinations.

DISCUSSION

The recovery of total radioactive carbon dioxide in the expired air following the administration of a tracer dose of labeled fatty acid has been previously used as an index to the rate of metabolization in animals (5, 9, 10) and humans (2, 4). Using carbon 14-labeled palmitic acid, Brown was able to recover CO_2 within 2 min after intravenous injection in human volunteers (2). This rapid recovery of $C^{14}O_2$, which was also noted with the short chain fatty acid used in our experiments, is advantageous in the study of fatty acid absorption and oxidation.

In our studies $C^{14}O_2$ appeared in the expired air in 1–2 min after intravenous and 3–6 min after oral administration of the isotope. The difference between these two periods, i.e., the delay time after the oral dose minus that after the intravenous dose, represents the transit time of the isotope from the mouth to the portal venous system. Inasmuch as no significant difference in the recovery of $C^{14}O_2$ after oral and intravenous administration was noted, it would seem that the small amount of RAO used in these studies is completely absorbed from the intestinal tract. For this reason it would appear that the recovery of orally administered RAO may be used as an index of absorption in individuals without evidence of defective oxidation of fatty acids. Many studies in recent years have pointed out that the absorption transport and metabolism of fatty acids with 10 or less carbon atoms is handled differently than those with more than 10 carbon atoms (4, 6, 7). A number of experiments have determined *a*) that fatty acid absorption decreases as the molecular weight of the fatty acid increases, *b*) that fatty acids with one or more double bonds are absorbed to a greater degree than are their saturated counterparts, and *c*) that the degree of absorption of any given fatty acid is greater if it is mixed with less well-absorbed fatty acid (1, 3). These factors must be taken into consideration in determining whether octanoic acid absorption may be taken as indicative of normal fat absorption. Further studies are needed before this question can be answered fully.

Inhibition of long-chain fatty acid oxidation by glucose has been previously demonstrated by similar techniques (2, 7). Lossow and Chaikoff observed a marked inhibition of palmitic acid oxidation in rats after feeding a diet consisting of glucose, lactalbumin, corn oil, salt, and a vitamin mixture.

In the case of octanoic acid, however, only a minimal degree of inhibition was noted. Our studies performed after the administration of glucose, in an amount used in the standard glucose tolerance test, failed to reveal any significant inhibition of octanoate oxidation by this technique.

Intravenous infusion with moderate doses (μg) of unlabeled octanoic acid likewise failed to inhibit the oxidation of C^{14}-labeled octanoic acid. It should be emphasized that with this 1-g dose of octanoic acid no significant change occurred in the shape of the curve or the location of the peak, as well as the total amount of $C^{14}O_2$ recovered in 50 min. This failure of inhibition of RAO oxidation may be due to the moderate dose of octanoic acid used or to the slow rate of infusion, i.e., approximately 17 mg/min. A larger dose, 2 g, of octanoic acid, however, was accompanied by flushing, nausea, joint and back pains in one volunteer, and resulted in a moderate depression of RAO oxidation. Because of these disturbing side effects no additional subjects were tested with this larger dose. The results suggest that oxidation occurred at the same rate after the 1-g octanoate infusion as with the tracer dose (149 μg) alone. We know from previous experiments that octanoate is rapidly metabolized (7), but the exact maximum rate of oxidation has not been clearly established in humans.

The studies reported here suggest that small doses of octanoic acid are virtually completely absorbed shortly after ingestion and that prompt oxidation is not substantially inhibited by moderate doses of glucose by mouth or by intravenous fatty acid. These features of octanoic acid metabolism could be exploited in the clinical study of fat absorption.

The authors are grateful to Binnie Rem for valuable technical assistance.

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ÉTUDE «*IN VITRO*» DE L'OXYDATION DES ACIDES GRAS PAR DIVERS TISSUS DU JEUNE RAT SOUMIS A UN RÉGIME STEATOGÈNE (HYPOPROTÉIQUE, HYPERLIPIDIQUE) PRIVÉ DE CHOLINE ET DE VITAMINE B_{12}

II. LE TISSU HÉPATIQUE

par

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(*Reçu le 27 octobre 1966*)

INTRODUCTION

Dans un précédent travail (4) nous avons montré que le jeune rat soumis dès le sevrage à un régime hypoprotéique, hyperlipidique, privé de choline et de vitamine B_{12}, ne présente dans le rein aucun des signes de la carence en choline signalée par de nombreux auteurs. La signification de ce résultat a été discutée.

Dans le présent mémoire, nous étudions les effets de ce même régime au niveau du foie. Les recherches portent essentiellement sur les mesures de l'activité du système d'oxydation des A. G.

RECHERCHES PERSONNELLES

1° Conditions expérimentales et techniques utilisées

Les expériences utilisent des rats mâles de 22 jours, pesant environ 40 grammes, de souche Wistar.

Les animaux sont divisés en 2 lots : le premier reçoit le régime complet (1) et le deuxième le régime stéatogène, privé de choline et de vitamine B_{12}. La composition de ces régimes a été donnée précédemment (2).

Après 28 jours de régime les animaux sont tués par décapitation, le foie est rapidement prélevé et homogénéisé. L'activité de l'octanoate-oxydase est mesurée dans les mêmes conditions que pour le rein (3) : par la consommation d'extra-oxygène et par le dosage de l'octanoate disparu. Nous avons, de plus, déterminé les corps cétoniques formés par la technique de WEISCHELBAUM et SOMOGYI (4).

RÉSULTATS EXPÉRIMENTAUX ET DISCUSSION

Les résultats exprimant la correspondance entre la consommation d'oxygène utilisé pour l'oxydation de l'octanoate et la quantité d'octanoate disparu sont résumés dans le tableau I.

TABLEAU I

Activité octanoxydasique de l'homogénat de foie de rat mâle Wistar âgé de 8 semaines

(1) Régime des rats	(2) Nombre d'expériences	(3) Consommation de O_2 en 20' (µM/mg N)			(4) Octanoate disparu	(5) Rapport extra O_2 consommé / Oct. disparu
		T	E	T-E		
1	7	2,52 (± 0,19)	1,24 (± 0,13)	1,28 (± 0,11)	0,86 (± 0,10)	1,50 (± 0,10)
2	11	2,54 (± 0,15)	1,67 (± 0,07)	0,87 (± 0,07)	0,74 (± 0,05)	1,09 (± 0,04)

L'examen de ce tableau met en évidence les faits suivants :

1° L'oxygène consommé pour l'oxydation de l'oxtanoate (T — E) présente des valeurs plus faibles chez le jeune rat soumis au régime stéatogène que chez les témoins : 1,28 (régime 1) et 0,87 (régime 2), ceci représente une diminution de l'activité de l'octanoate-oxydase de l'ordre de 30 p. 100. Ce résultat montre que chez le jeune rat (*), comme chez l'adulte, la carence en choline s'accompagne d'une diminution du pouvoir oxydasique du foie à l'égard des A. C. Les diminutions observées chez l'adulte étaient toutefois plus importantes puisqu'elles étaient de l'ordre de 60 à 80 p. 100;

2° Alors que chez l'adulte la valeur du rapport $\frac{\text{extra-oxygène consommé}}{\text{octonoate disparu}}$ était de l'ordre de trois, correspondant à la transformation de l'octanoate en composés cétoniques, chez le jeune rat ce même rapport est égal à 1,50 pour les animaux témoins et 1,09 pour ceux soumis au régime stéatogène.

Ces valeurs ne correspondent pas à la transformation de l'octanoate disparu en composés cétoniques (rapport théorique 3) et encore moins à son oxydation totale (rapport théorique 11).

Pour discuter la signification de ce résultat nous avons mesuré la quantité de composés cétoniques formés correspondant aux quantités d'octanoate disparu. Les résultats sont réunis dans le tableau II.

TABLEAU II

Mesure de la formation de composés cétoniques correspondant à l'oxydation de l'octanoate par l'homogénat de foie de rat mâle Wistar âgé de 8 semaines

(1) Régime des rats	(2) Nombre d'expériences	(3) Octanoate disparu en 20' (µM mg N)	(4) C. cétoniques formés en 20' (µM mg N)	(5) Rapport C. Cétoniques / Oct. disparu
1	5	1,12 (± 0,07)	0,95 (± 0,09)	0,85 (± 0,08)
2	7	0,81 (± 0,08)	0,65 (± 0,08)	0,80 (± 0,06)

L'examen de ce tableau montre que la moitié environ de l'octanoate disparu s'est transformé en composés cétoniques.

(*) Nous avons vérifié que ces jeunes rats présentent également la stéatose hépatique.

Si donc seulement 50 p. 100 de l'octanoate disparu a été transformé en composés cétoniques, le problème se pose évidemment de connaître la destinée des 50 autres p. 100 disparus. Ont-ils subi une oxydation totale? S'il en était ainsi, le rapport $\frac{\text{extra-oxygène consommé}}{\text{octonoate disparu}}$ devrait être égal à 7, ce qui n'est point le cas puisque, comme il a été dit plus haut, ce rapport calculé à partir de l'extra-oxygène mesuré est de l'ordre de 1. Or, même en admettant que l'extra-oxygène mesuré ne corresponde pas à l'oxydation de l'octanoate disparu et en supposant que l'octanoate se substitue aux substrats de la respiration endogène, le rapport $= \frac{\text{oxygène consommé}}{\text{octonoate disparu}}$ calculé alors à partir de l'oxygène total consommé, est égal à 3. Ce chiffre est bien éloigné du 7 théorique. On ne peut retenir l'hypothèse selon laquelle les 50 p. 100 de l'octanoate disparu non transformés en corps cétoniques auraient subi une oxydation totale.

De plus, on constate que les valeurs théoriques d'oxygène nécessaires pour transformer 50 p. 100 de l'octanoate disparu en composés cétoniques correspondent aux valeurs expérimentales mesurées en prenant l'extra-oxygène comme étant la quantité d'oxygène utilisée pour effectuer cette transformation. En effet, chez les animaux soumis au régime stéatogène, la quantité expérimentale d'extra-oxygène est de = 0,970 µM en 20 minutes, alors que la quantité théorique est de 0,975 µM dans ce cas. De même, chez les animaux témoins, la quantité expérimentale d'extra-oxygène est de = 1,740 µm en 20 minutes alors que la quantité théorique d'oxygène nécessaire est ici de = 1,425 µM.

Cet ensemble de faits nous amène à conclure que la moitié environ de l'octanoate disparu s'est transformé en composés cétoniques, sans qu'il soit possible à l'heure actuelle de préjuger de la destinée de l'autre moitié disparue.

CONCLUSIONS

1° L'étude de l'oxydation de l'octanoate par l'homogénat de foie du jeune rat montre que seulement 50 p. 100 environ de l'octanoate disparu se transforme en composés cétoniques.

2° L'administration du régime hypoprotéique, hyperlipidique, privé de choline et de vitamine B_{12} a pour conséquence de diminuer l'intensité de l'oxydation de l'octanoate dans le tissu hépatique. Celle-ci présente toutefois les mêmes caractéristiques que chez l'animal témoin.

3° La signification de ces résultats est discutée.

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Recherches sur l'action inhibitrice des acides gras et, en particulier des acides non saturés, sur les phénomènes diastasiques,

par M. L. VELLUZ.

(7 mars 1927).

Dans un mémoire relatif au mécanisme d'action des diastases protéolytiques, MM. L. Hugounenq et J. Loiseleur [1] ont montré que, des deux éléments nécessaires aux phénomènes diastasiques étudiés, l'un — la micelle colloïdale — sert de vecteur à l'autre — cristalloïde ionisé — par sa polarité propre qui est susceptible de créer une électivité dans l'adsorption ionique. La suppression de l'un des facteurs entraine la disparition du phénomène diastasique : en l'absence d'électrolytes, la pepsine et la trypsine sont inactives ; en présence d'un colloïde inerte (gomme arabique) la protéolyse par les électrolytes (acide chlorhydrique, carbonate de sodium) n'est sensiblement pas modifiée [2]. Avec le glycogène, doué d'une polarité faible, on observe cependant une action catalytique incontestable.

Les recherches dont nous exposons les résultats dans ce mémoire montrent qu'on parvient à diminuer l'activité protéolytique de la pepsine en précipitant à la surface de sa micelle un acide gras, et en particulier un acide non saturé. Il est permis de supposer que cette action inhibitrice est due à la formation d'un film périmicellaire inerte qui diminue considérablement la polarité de la micelle ainsi enrobée. Nous avons obtenu des résultats analogues avec l'uréase du Jack Bean (*Canavalia ensiformis*).

∴

Si on dissout le sel sodique d'un acide gras dans une solution colloïdale quelconque (gomme arabique, glycogène, pepsine, trypsine, uréase) une fraction variable du savon est adsorbée à la surface des micelles. Cette adsorption s'exprime mathématiquement.

Cap Acid

par la loi de Gibbs, applicable à toute substance qui modifie la tension superficielle d'un liquide :

$$\Gamma = -\frac{c}{RT} \times \frac{\delta\sigma}{\delta c}$$

Γ = Accumulation du corps dissous par unité de surface de la couche superficielle :
σ = Tension superficielle du solvant ;
c = Concentration du corps dissous dans le solvant ;
RT = Constante des gaz parfaits.

Dans le cas d'un savon, $\frac{\delta\sigma}{\delta c}$ est négatif : l'adsorption représentée ici par Γ est donc positive, ce qui revient à dire que le savon se concentre autour de chaque micelle colloïdale. Cette adsorption croît avec la température. Par addition de NaCl, il y a floculation du complexe colloïde-savon.

Nous avons recherché si cette adsorption d'un savon à la surface d'une micelle diastasique en modifie l'activité. Dans le cas de la pancréatine et de l'uréase, nous n'avons observé aucune modification.

Pour obtenir la formation d'un complexe colloïde-acide gras, il suffit d'acidifier dans une proportion convenable une solution colloïde-savon. L'acide gras libéré, s'il est insoluble ou très peu soluble dans l'eau, reste fixé sur la micelle colloïdale par un phénomène d'ordre vraisemblablement mécanique. La précipitation du colloïde par ses agents habituels de floculation entraîne l'acide gras fixé sur la micelle. La solution colloïde-acide gras présente une opalescence variable avec l'acide gras employé et surtout avec la concentration de cet acide. Pour éviter une floculation partielle ou totale du support colloïdal, il ne faut pas dépasser le plus souvent la concentration en acide gras de 20 p. 100 de colloïde sec.

Nous avons ainsi préparé des solutions colloïdales diastases-acides gras, stables et de concentrations variables en diastases et acides gras. Pour l'étude quantitative des enzymes ainsi traitées, nous avons dû opérer en milieu acide pour éviter la solubilisation de la pellicule. De plus, la nécessité d'une titration précise nous a conduit à opérer sur la pepsine et l'uréase.

Les acides gras utilisés ont été les suivants :

1° *Acides saturés* : Acides caproïque, œnanthylique, caprylique, pélargonique, caprique, palmitique, stéarique.

2° *Acides non saturés* : Acides crotonique, diméthylacrylique, oléique, ricinoléique. Acides non saturés de l'huile de lin, préparés selon la méthode indiquée par G. Bertrand [3] et que nous désignerons par convention : acide linolénique (indice d'iode 188).

I. — Etude de la pepsine.

Nos essais ont été faits sur la pepsine commerciale dite « en paillettes » de titre 1.000. Les digestions ont été faites à 37° sur de l'ovalbumine fraîche, coagulée par la chaleur et divisée en cubes sensiblement identiques. Nous n'avons pas employé l'ovalbumine colloïdale pour éviter les phénomènes d'adsorption secondaire susceptibles de modifier l'interprétation de nos résultats. L'azote aminé soluble, libéré par la protéolyse dans un temps variant de 6 à 16 heures a été déterminé selon la technique de Sörensen au formol.

A. — *Forte concentration en acides gras par rapport à la concentration en pepsine :*

Pepsine	0,5 p. 1000
Acide chlorhydrique	0,5 —
Ovalbumine coagulée	5 p. 100
Acides gras	3 p. 1000

	Azote soluble libéré après 16 h. d'étuve (1)
Témoin	4,3
— + acide caproïque	4,2
— — œnanthylique	3,1
— — caprylique	3,1
— — pélargonique	2,3
— — caprique	2,1
— — palmitique	1,1
— — stéarique	1
— — oléique	1
— — linolénique	1
— — ricinoléique	1

Tous les acides gras diminuent donc le pouvoir protéolytique de la pepsine. L'action inhibitrice croît avec la condensation en carbone, de C^6 à C^{18}.

Il est naturel d'attribuer ce fait à la solubilité correspondante

(1) En mgr. p. 100 cm³ de liquide essayé.

des acides dans l'eau, solubilité qui décroît aussi quand la teneur en carbone s'élève. Si la micelle colloïdale est enrobée par un acide gras légèrement soluble dans l'eau, le film périmicellaire se prête plus facilement à des échanges par diffusion ou osmose, échanges qui conditionnent l'action diastasique.

A même condensation en carbone — C^{18} —, aucune différence n'est observée entre acides saturés ou non saturés.

Toutefois, cet essai est, à lui seul, peu probant par suite de l'instabilité du colloïde pepsine-acides gras aux concentrations considérées. Après 16 heures d'étude une floculation partielle se produit et le simple entraînement de la pepsine pourrait expliquer à lui seul la lenteur de la protéolyse. Néanmoins les variations relatives dans cette floculation sont déjà susceptibles d'être interprétées.

B. — *Faible concentration en acides gras par rapport à la concentration en pepsine* :

Nous avons employé dans ce cas deux échantillons de pepsine de titres 100 et 1000 et nous avons remarqué que plus le titre d'une pepsine est faible, plus la concentration en acides gras peut être forte sans qu'il y ait floculation.

Théoriquement, en effet, si l'adsorption de l'acide par la micelle pepsique, est un phénomène d'ordre électrostatique, il ne peut y avoir formation du colloïde pepsine-acides gras dont les constituants sont tous deux électronégatifs. Pour une pepsine de titre faible, la présence de colloïdes étrangers, sans polarité propre, permet la fixation des acides gras, d'où l'apparence d'une concentration limite bien supérieure. Pour une pepsine de titre très élevé, la formation du complexe d'adsorption est vraisemblablement due à un phénomène purement mécanique, dont l'intensité n'est jamais comparable à celle d'un phénomène d'ordre électrique.

Pepsine	2,5 p. 1000
Acide chlorhydrique	2,5 —
Ovalbumine coagulée	5 p. 100
Acides gras	0,4 p. 1000

	Azote soluble libéré après	
	6 h. d'étuve peps. titre 1000	18 h. d'étuve peps. titre 100
Témoin	6,5	7,2
— + acide caproïque	6,4	6,6
— — œnanthylique	6,4	6,6

Témoin + acide caprylique	6,2	6,3
— — pélargonique	5,9	6,3
— — caprique	5,9	6,1
— — palmitique	5,6	5,8
— — stéarique	5,6	5,8
— — oléique	5,1	2,8
— — ricinoléique	3,6	1,2
— — linolénique	3,7	1,3

Nous retrouvons ici la relation entre le pouvoir d'inhibition d'un acide gras et sa teneur en carbone ; mais, contrairement à l'essai précédent, pour une même teneur en carbone — C^{18} — il y a une différence entre acides saturés et non saturés. Nous croyons pouvoir interpréter ce fait en faisant intervenir les théories de Langmuir [4] relatives à l'adsorption et à l'étalement des acides gras.

Selon cet auteur :

1° La couche d'adsorption d'un corps à la surface de séparation de deux phases est unimoléculaire ;

2° Pour un acide gras insoluble dans l'eau, la surface recouverte par cette couche unimoléculaire (force d'étalement) est fonction de la présence dans sa molécule de groupements chimiques déterminés.

Langmuir a ainsi déterminé la surface moléculaire de plusieurs acides gras en C^{16} et C^{18}, et ses résultats sont les suivants :

Surface couverte par une molécule d'acide palmitique	21×10^{-16} cm²
— stéarique	22×10^{-16} —
— oléique	46×10^{-16} —
— linolénique	65×10^{-16} —
— ricinoléique	110×10^{-16} —

En présence d'acide chlorhydrique à 0,1 p. 100, les surfaces sont légèrement augmentées :

Acide palmitique	37×10^{-16}
— stéarique	35×10^{-16}
— oléique	67×10^{-16}
— ricinoléique	126×10^{-16}

Les acides non saturés couvrent tous un espace plus grand, par molécule, que les acides saturés. La molécule de l'acide ricinoléique apparaît comme la plus étendue, par suite de la présence de trois groupes « actifs » : COOH, OH alcoolique, liaison éthylénique.

Considérons alors une micelle colloïdale partiellement enrobée par un acide gras : pour une même concentration de cet acide l'enrobement sera d'autant plus parfait que l'on passera de l'acide stéarique à l'acide ricinoléique. Il en sera de même pour l'ensemble des surfaces micellaires. Le pouvoir inhibiteur de l'acide variera dans le même sens que sa capacité d'étalement.

Nos résultats vérifient tout à fait cette hypothèse dans le cas de la pepsine. Toutefois, les théories de LANGMUIR ne sont rigoureusement applicables que dans des conditions physico-chimiques bien déterminées qui sont loin d'être les nôtres. La complexité des facteurs dans les essais biologiques ne permet de rechercher qu'un ordre de grandeur dans l'étude des phénomènes. C'est seulement cet ordre de grandeur que nous cherchons à retrouver dans le parallélisme entre le pouvoir inhibiteur et la force d'étalement.

Si la concentration de l'acide gras est telle qu'avec l'acide de force d'étalement minima toutes les micelles sont enrobées, on ne doit plus observer de différences entre acides saturés et non saturés, exception faite des variations possibles dues à des états physiques dissemblables. C'est le cas du premier essai mentionné plus haut.

C. — *Influence de la concentration en acide gras :*

Essai I. : Pepsine..... 0,5 p. 1000
Acide chlorhydrique..... 0,5 —
Ovalbumine coagulée..... 5 p. 100

	Azote libéré après 16 heures d'étuve
Témoin.....	6,1
— + acide oléique 0,2 p. 1000.....	5,3
— + — 0,5 —	5
— + — 3 —	0,8 (floculation partielle).

Essai II. : Pepsine..... 3 p. 1000
Acide chlorhydrique ..... —
Ovalbumine coagulée ..... 5 p. 100

	Azote libéré après 6 heures d'étuve
Témoin.....	10,9
— + acide palmitique... 2 p. 1000.....	10,3
— + acide ricinoléique.. 2 —	6,5
— + — .. 10 —	0,4 (floculation).

Le pouvoir inhibiteur croît avec la concentration en acide ; pour la concentration de 2 p.1.000 on observe toujours une différence notable entre l'acide palmitique et l'acide ricinoléique.

Les essais suivants montrent que la concentration en acide chlorhydrique ne modifie pas l'allure du phénomène.

Essai : Pepsine..... 3 p. 1000 (titre 100).
Ovalbumine coagulée..... 5 p. 100
Acide ricinoléique ..... 1 p. 1000

	Azote libéré après 20 heures d'étuve	
	acide chlorhydrique 3 p. 1000	acide chlorhydrique 6 p. 1000
Témoin	9,1	9,3
— + acide ricinoléique	3,1	2,9

Nous avons observé de plus qu'en l'absence d'électrolytes la fixation d'acides gras sur la micelle pepsique ne lui confère aucun pouvoir protéolytique. De même, la présence d'un colloïde, gomme-arabique-acide gras, ne modifie pas la protéolyse par les électrolytes seuls, ce qui paraît montrer que l'enrobement par une pellicule d'acides gras ne confère à la micelle aucune polarité propre.

Si l'acide gras est fixé sur une micelle autre que la micelle diastasique (gomme arabique), sa présence ne modifie pas la digestion pepsique :

	Azote libéré après 20 heures d'étuve
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) 3 p. 100 + acide chlorhydrique 3 p. 100	8,2
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) + gomme arabique 2 p. 100.....	8,1
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) + gomme arabique 2 p. 100 + acide oléique 3 p. 1000.....	7,9
Ovalbumine coagulée 5 p.100 + pepsine (titre 100) + gomme arabique 2 p. 1000 + acide ricinoléique 3 p. 1000.....	7,9
Ovalbumine coagulée 5 p. 100 + pepsine (titre 100) + gomme arabique 2 p. 100 + acide linolénique 3 p. 1000.....	7,9

Enfin si l'acide considéré est soluble dans l'eau, il n'influence en rien l'activité protéolytique :

	Azote libéré après 6 h. d'étuve
Ovalbumine coagulée 5 p. 100 + pepsine (titre 1000) + acide chlorhydrique 1 p. 1000	6,2
Ovalbumine coagulée 5 p. 100 + pepsine (titre 1000) + acide crotonique 1 p. 1000	6,1
Ovalbumine coagulée 5 p. 100 + pepsine (titre 1000) + acide diméthylacrylique 1 p. 1000	6,2

D.— *Fixation de l'acide gras sur l'albumine en digestion.*

La technique suivie pour l'obtention du colloïde ovalbumine-acide-gras est un peu différente de celle donnée plus haut pour la pepsine. Nous avons préparé une solution alcaline d'ovalbumine.

Ovalbumine fraiche	50 cm³
Soude normale, q. s. p.	250 —

Le savon a été dissous dans cette solution alcaline et nous avons ajouté de l'acide chlorhydrique en quantité suffisante pour saturer la soude et décomposer le savon.

Essai I. : Pepsine	1 p. 1000 (titre 100)
Acide chlorhydrique	—
Ovalbumine colloïdale	3 p. 100
Acides gras	2 p. 1000

	Azote libéré après 20 h. d'étuve
Témoin	13,2
— + acide caproïque	11,9
— — oenanthylique	11,8
— — caprylique	11,8
— — caprique	11,2
— — palmitique	11,2
— — stéarique	11,2
— — oléique	11
— — linolénique	10,3
— — ricinoléique	10,3

Essai II (sans pepsine) + Acide chlorhydrique	3 p. 1000
Ovalbumine colloïdale	1 p. 100
Acides gras	2 p. 1000

	Azote libéré après 20 h. d'étuve
Témoin	2 mgr.,1
— + acides gras	1 mgr.,6

Ces résultats sembleraient donc montrer que la protection de l'albumine par les acides gras est d'autant plus accentuée que les acides sont plus riches en carbone ou non saturés. Mais, en raison des faibles différences observées, nous ne croyons pas devoir en faire état.

II. — Etude de l'uréase.

Le dynamisme de la diastase du Jack Bean (*Canavalia ensiformis*), étudié par Van Slyke [5], paraît être différent de celui de la pepsine. En effet, selon cet auteur, l'hydratation de l'urée par l'uréase comporte deux phases successives :

1° Combinaisons de l'enzyme avec l'urée :

$$[E] + [U] \longrightarrow [EU] \qquad (1)$$

réaction de vitesse constante c ;

2° Rupture de cette combinaison, l'urée étant libérée sous forme de carbonate d'ammoniaque

$$[EU] \longrightarrow [E] + CO^2 + NH^3 \qquad (2)$$

réaction de vitesse constante d.

L'application de la loi de Guldberg et Waage aux équations (1) et (2) a permis à Van Slyke de formuler la loi mathématique suivante

$$t = \frac{1}{E}\left[\frac{1}{c}\,\mathrm{Log.}\,\frac{a}{a-x} + \frac{x}{d}\right]$$

où t est le temps de décomposition d'une quantité x d'urée, E la concentration de l'enzyme et a la quantité d'urée présente par unité de volume au début de la réaction.

L'expérience vérifie parfaitement cette formule si on prend toutefois la précaution d'opérer en présence de solutions tampons qui maintiennent le pH constant. Les vitesses de réactions c et d varient en effet beaucoup avec le pH ; elles passent par un maximum pour pH = 7 et tendent vers zéro quand l'alcalinité du milieu augmente par suite de l'accumulation du carbonate d'ammoniaque formé.

Nous avons préparé une uréase d'une activité suffisante par simple macération de 10 gr. de farine de Jack Bean dans 100 gr. d'eau distillée à 15°-20° pendant 24 heures, sous toluène. La

solution obtenue après centrifugation constitue une solution mère dont nous avons utilisé une partie aliquote dans nos essais.

L'acide gras a été fixé sur la micelle d'enzyme selon la même technique que pour la pepsine. Le colloïde uréase-acide gras, précipite par addition de 5 fois son volume d'acétone et le floculat renferme intégralement l'acide fixé.

Les solutions utilisées ont été uniformément amenées au pH = 4,7, par dilution convenable d'acide acétique normal et acétate de soude normal, à parties égales. Dans tous nos essais le pH n'a pas varié de plus de 0,15, du début à la fin de la fin de la réaction. Chaque solution mère n'ayant été utilisée que pour une série d'essais, nos résultats ne doivent pas être considérés en valeur absolue, mais seulement en valeur relative par rapport aux témoins correspondants.

Le dosage de l'ammoniaque formé a été effectué, sur 100 cm³ de liquide essayé, selon la méthode au formol.

I. — *Même concentration en uréase et acides gras :*

Essai : Uréase..... 0,5 p. 1000
Urée..... 5 p. 100
Acides gras..... 0,5 p. 1000

	Ammoniaque formée en 30' (1)
Témoin	52
— + acide linolénique	48
— — ricinoléique	42
— — oléique	46
— — stéarique	36
— — palmitique	36

Il y a inhibition de l'enzyme comme dans le cas de la pepsine, mais ici l'allure du phénomène est inversée : l'inhibition est plus forte pour les acides saturés — solides — que pour les acides non saturés liquides. Nous attribuons ce fait au dynamisme particulier de l'uréase : première phase où l'enzyme se combine avec le substrat. Cette combinaison est vraisemblablement plus difficile en présence d'une pellicule solide, moins sensible à l'osmose qu'une pellicule liquide.

II. — *Faible concentration en acides gras par rapport à la concentration en uréase :*

(1) En mgr. p. 100.

Essai : Uréase..... 2,5 p. 1000
Urée..... 5 p. 100
Acides gras..... 0,2 p. 1000

	Ammoniaque formée en 30'
Témoin	114
— + acide palmitique	114
— — stéarique	114
— — oléique	98
— — ricinoléique	88

Le parallélisme entre le pouvoir inhibiteur et la force d'étalement réapparait comme dans le cas de la pepsine.

III. — *Rôle de la concentration en acide gras :*

Essai : Uréase..... 1 p. 1000
Urée..... 1 p. 100

	Ammoniaque formée en 30' — Concentration p. 1000 en acide gras					
	0,2	0,1	0,08	0,04	0,02	
Acide ricinoléique	33	38	38	36	42	
— oléique	35	39	40	42	42	témoin
— linolénique	32	34	35	39	42	42 mgr.
— palmitique	40	39	40	42	42	
— stéarique	40	39	40	42	42	

La concentration minima où le pouvoir inhibiteur apparait, est en relation avec la force d'étalement.

Les résultats donnés par les essais précédents sont donc sensiblement symétriques de ceux obtenus avec la pepsine. Il importe toutefois de remarquer que dans le cas de l'uréase le phénomène est plus complexe que dans le cas de la pepsine. Ici, trois facteurs interviennent :

1° Etat physique, solide ou liquide, de l'acide employé ;
2° Force d'étalement de l'acide ;
3° Pression osmotique de la solution d'urée.

Ce dernier facteur nous a paru particulièrement important. Si on imagine, en effet, une micelle enrobée d'une pellicule insoluble, mais cependant sensible à la pression osmotique, les échanges à travers cette pellicule sont fonction :

1° de son épaisseur ;

2° de la pression osmotique du liquide extérieur.

Or, pour une même concentration en acide (1), l'épaisseur d'une pellicule est d'autant plus faible que la force d'étalement est plus forte. Ainsi, les échanges par osmose seront plus faciles pour une pellicule d'acide ricinoléique que pour une pellicule d'acide oléique. Les 2 facteurs, force d'étalement et épaisseur de la pellicule, ont deux effets distincts : film périmicellaire mince, mais enrobant un nombre important de micelles, ou film périmicellaire de plus forte section, mais n'enrobant qu'un petit nombre de micelles. Il est dès lors permis de supposer que, selon la pression osmotique du liquide extérieur — c'est-à-dire la concentration en urée —, le pouvoir inhibiteur variera soit dans le même sens que la force d'étalement, soit en sens contraire.

Nous avons fait dans ce but une série d'essais où la concentration de l'urée varie de 0,1 à 12 p. 100.

Essai : Urease..... 1 p. 1000
Acides gras..... 0,4 p. 1000
Temps..... 30 minutes.

Concentration en urée p. 100	Ammoniaque formée (*)			
	Témoin	Acide palmitique	Acide oléique	Acide ricinoléique
12	29,1	29	27,3	25,6
10	29,1	29	27,3	26,6
5	29,1	27,1	24,2	21,3
1	23	21,2	19,4	20,3
0,5	19	18,4	15,6	16,8
0,2	12	11,1	9,7	10,5
0,1	7,1	6,8	6,3	6,6

(*) Exprimé ici en cm³ de soude décinormale.

(1) Concentration faible par rapport à la concentration en diastase.

D'après ce tableau, on voit que :

1° Avec une concentration en urée au-delà de 1 p. 100, la pression osmotique est assez forte pour que l'épaisseur du film périmicellaire n'intervienne plus. L'action inhibitrice croît alors avec la surface totale enrobée, c'est-à-dire avec la force d'étalement.

2° Avec une concentration en urée égale ou inférieure à 1 p. 100, l'épaisseur du film intervient suffisamment pour que l'acide oléique devienne plus inhibiteur que l'acide ricinoléique.

Nous avons tenté d'appliquer à l'essai précédent l'équation de Van Slyke

$$t = \frac{1}{E}\left[\frac{1}{C}\ \text{Log.}\ \frac{a}{a-x} + \frac{x}{d}\right]$$

qui peut encore s'écrire :

$$0{,}4343\ c = \frac{d}{Et\,d-x}\ \text{Log.}\ \frac{a}{a-x}$$

La constante d est déduite des valeurs de x et t déterminées quand la concentration de l'urée est assez forte pour que la vitesse de réaction en soit indépendante : $\frac{1}{c}\log.\frac{a}{a-x}$ devient négligeable

$$t = \frac{1}{E} \times \frac{x}{d} \qquad d = \frac{x}{Et}$$

on remplace, dans l'équation de Van Slyke, les lettres par leurs valeurs numériques et on recherche la constance de C.

Les valeurs de c obtenues dans les 4 tableaux suivants montrent que si l'équation de Van Slyke est applicable pour l'essai témoin, elle ne l'est plus quand l'uréase est enrobée par un acide gras. La cinétique de la réaction est modifiée, surtout aux concentrations fortes en urée. C'est dans le cas d'un film périmicellaire de faible épaisseur (ac. ricinoléique) que la vitesse c (vitesse de combinaison de l'enzyme avec l'urée) reste sensiblement constante.

***

En résumé, alors que la fixation d'un savon sur une micelle diastasique telle que la pancréatine ou l'uréase reste sans effet appréciable sur son activité, la fixation d'un acide gras sur la pepsine et l'uréase la modifie notablement. Dans certaines limites de concentration, les acides non saturés sont plus actifs que les

1) Essai : témoin.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	29,1	9,7	
10	3330	29,1	9,7	
5	1665	29,1	9,7	
1	333	23		0,498
0,5	166,5	19		0,499
0,2	66,6	12		0,487
0,1	33,3	7,1		0,460
	exprimés en cm3 soude décinormale			

2) Essai : témoin + acide palmitique.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	29	9,66	
10	3330	29	9,66	
5	1665	27,1		0,513
1	333	21,2		0,37
0,5	166,5	18,4		0,455
0,2	66,6	11,1		0,426
0,1	33,3	6,8		8,43
	exprimés en cm3 soude décinormale			

3) Essai : témoin + acide oléique.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	27,3	9,1	
10	3330	27,3	9,1	
5	1665	21,2		0,417
1	333	19,4		0,287
0,5	166,5	15,6		0,319
0,2	66,6	9,7		0,313
0,1	33,3	6,3		0,414
	exprimés en cm3 soude décinormale			

4) Essai : témoin + acide ricinoléique.

Concentration en urée p. 100	a	x	d	0,4343 c
12	3996	25,6	8,53	
10	3330	25,6	8,53	
5	1665	21,3		0,24
1	333	20,3		0,40
0,5	166,5	16,8		0,43
0,2	66,6	10,5		0,42
0,1	33,4	6,6		0,43
	exprimés en cm3 soude décinormale			

acides saturés et ce fait parait en relation avec leur force d'étalement.

L'intérêt de ces résultats nous parait dépasser celui d'une expérimentation réalisée *in vitro*. En effet, par l'antagonisme du pH des liquides interstitiels (pH 7,3), et cellulaire, (pH 6), une fixation analogue à celle que nous considérons ici est possible *in vivo*.

Il est permis d'imaginer l'existence de complexes diastases-savons dans les liquides de pH > 7 et celle de complexes diastases-acides gras dans les liquides de pH < 7. Dans les conditions normales du métabolisme des acides gras, il est probable que l'association enzyme-acide contribue à l'équilibre diastasique. Ne peut-on pas alors supposer que la déviation de ce métabolisme (combustion ou production excessives, prédominance des acides non saturés sur les acides saturés) modifiant l'activité des complexes diastases-acides gras alors qu'elle affecte peu celles des complexes diastases-savons, il y aura dysharmonie dans le jeu diastasique complet ?

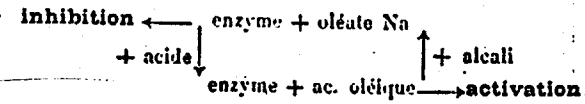
MM. Hugounenq et Loiseleur [6] ont montré que le chimisme d'une micelle colloïdale amphotère peut présenter des actions diastasiques inverses pour des variations du pH de part et d'autre du point isoélectrique. Dans le cas des associations enzymes-savons enzymes-acides gras, l'activité diastasique pourra être modifiée sans variations du pH. Il est d'ailleurs possible que l'existence de ces couples diastasiques constitue pour l'organisme un mécanisme régulateur en quelque sorte « tampon » du système diastasique. On peut schématiser ce fait de la façon suivante :

1° pH constant (< 7) :

Solution tampon : acide oléique + oléate de soude

$$\text{acide oléique} \underset{+\ \text{acide}}{\overset{+\ \text{alcali}}{\rightleftharpoons}} \text{oléate de soude} \quad (1)$$

En présence d'une micelle diastasique :



(1) Les flèches indiquent un déplacement de l'équilibre comme on le comprend en général dans les solutions tampons : les concentrations, des constituants sont toujours telles que la constance du pH est assurée.

2° pH variable (≷ 7) :

$$\text{enzyme} + \text{savon} \underset{\text{pH} > 7\ \text{(activation)}}{\overset{\text{(inhibition)}\ \text{pH} < 7}{\rightleftharpoons}} \text{enzyme} + \text{acide gras} \quad (1)$$

Nous suggérons l'intervention possible d'un mécanisme analogue dans l'équilibre ou le déséquilibre du biochimisme diastasique cellulaire et il est permis de croire à l'intérêt de recherches poursuivies dans cette voie, notamment en ce qui concerne la physiopathologie du cancer.

Quoiqu'il en soit, il apparait ici que les liaisons éthyléniques ont une importance considérable, quoique indirecte, dans le jeu des diastases associées *in vivo* à des acides gras ou à leurs sels. Nous avons déjà souligné le rôle qu'elles jouent vraisemblablement dans la narcose [7]. Alors que les lipides saturés constituent un potentiel énergétique d'ordre essentiellement *statique*, les acides non saturés paraissent jouer un rôle *dynamique* actif dans le métabolisme.

Conclusions.

1° Alors que l'adsorption d'un savon par une micelle diastasique telle que la pancréatine ou l'uréase reste sans effet sur son activité, la fixation d'un acide gras sur la pepsine et l'uréase modifie notablement cette activité.

2° Pour des concentrations en pepsine et en uréase, fortes par rapport à la concentration en acides gras, le pouvoir inhibiteur d'un acide gras croit avec sa concentration et s'accuse beaucoup plus avec les acides non saturés.

3° Dans le cas de l'uréase, deux facteurs interviennent pour modifier l'activité diastasique de l'enzyme ainsi enrobée : force d'étalement de l'acide et section du film périmicellaire. La pression osmotique du liquide extérieur conditionne alors l'effet résultant. La cinétique de la réaction ne permet plus d'appliquer la formule de Van Slyke.

(1) Les flèches n'indiquant plus ici un déplacement d'équilibre, mais le passage d'un constituant à l'autre.

4° Il est possible de supposer que l'équilibre ou le déséquilibre des jeux diastasiques *in vivo* est en partie sous la dépendance de couples diastasiques analogues.

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FATTY ACID METABOLISM. THE MECHANISM OF KETONE BODY SYNTHESIS FROM FATTY ACIDS, WITH ISOTOPIC CARBON AS TRACER*

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In a recent communication (1), results were reported of a study of the breakdown of *n*-octanoic acid labeled by incorporation of an excess of the heavy carbon isotope, C^{13}, in the carboxyl group. Proof was offered for a mechanism of ketone body formation involving the random coupling of identical 2-carbon units formed by β oxidation of the octanoic acid. The present paper is concerned with a more detailed description of these experiments and a discussion of the significance of the results obtained to the general question of the oxidation pathways of fatty acids.

EXPERIMENTAL

Preparation of Carboxyl-Labeled n-Octanoic Acid—Carbon dioxide was prepared by the oxidation of 0.49 gm. (0.01 mole) of isotopic sodium cyanide with aqueous potassium permanganate. The CO_2 was released by means of sulfuric acid into a high vacuum system, where it was purified and dried by repeated sublimation at −80° and condensation at −200°. Analysis in the mass spectrometer gave a C^{13} content of 5.51 per cent.[1] The CO_2 was then condensed into a highly evacuated flask containing an excess of *n*-heptylmagnesium bromide in ether. After several hours were allowed for the reaction to go to completion, the reaction mixture was decomposed with dilute sulfuric acid and distilled with steam. The distillate was extracted with ether, the ether extract was shaken with dilute sodium hydroxide, and the aqueous solution of sodium octanoate acidified and re-extracted with ether. The extract was then dried over anhydrous sodium sulfate and the ether evaporated. The residual colorless oil weighed 1.175 gm., representing a yield of 81 per cent. A Duclaux distillation revealed no perceptible volatile acid other than *n*-octanoic. Neutralization equivalent, observed, 143.0; calculated, 144.2.

[1] With the technical assistance of Mary Cammarotti.

[2] The C^{13} content is expressed as atoms per cent C^{13} calculated from the equation

$$\text{Atoms \% } C^{13} = \frac{\text{moles } C^{13}}{\text{moles } C^{12} + \text{moles } C^{13}} \times 100$$

The normal C^{13} complement for our instrument was found to be 1.01 per cent.

A specimen of this acid was oxidized according to the wet combustion method of Van Slyke and Folch (3). The resultant CO_2 had a C^{13} content of 1.59 per cent, corresponding to a C^{13} content for the carboxyl carbon of $(1.59 \times 8) - (1.01 \times 7) = 5.44$ per cent, in excellent agreement with the value of 5.51 per cent found for the C^{13} content of the original CO_2.

Incubation with Liver Slices—Liver slices of 0.4 mm. thickness from rats fasted 24 hours were shaken for 2 hours at 37.5° with 30 ml. of a 0.01 N sodium octanoate solution in a phosphate-buffered Ringer's solution. The composition of the medium was as follows: sodium octanoate 0.010 M, NaCl 0.051 M, PO_4 buffer, pH 7.4, 0.010 M, $CaCl_2$ 0.001 M, KCl 0.002 M. The reaction was carried out in Warburg type vessels of 125 ml. capacity in an atmosphere of oxygen, with 0.5 ml. of 2 N H_2SO_4 in the side cup and filter paper soaked with 0.5 ml. of 15 N NaOH in the central well. The uptake of oxygen was measured by an attached gas burette. At the end of the incubation period the acid, sufficient to bring the pH of the medium to 3.5, was tipped in from the side cup and shaking continued an additional 15 minutes to allow complete absorption of the liberated CO_2.

Determination of Respiratory CO_2—The alkali-soaked filter paper in the central well was quickly transferred to another Warburg vessel containing 20 ml. of CO_2-free water, the air was swept out with a stream of CO_2-free nitrogen, and, after equilibration, the CO_2 was liberated by acid tipped in from the side cup. The evolved CO_2 was measured volumetrically. The gases were then swept, by means of nitrogen, into an absorption tube containing barium hydroxide, from which the CO_2 was ultimately liberated by acid into an evacuated storage tube for determination of the C^{13} content.

Decomposition of Acetoacetic Acid and Isolation of Acetone and CO_2 Therefrom—The solution was decanted from the liver slices into a 50 ml. centrifuge tube, the slices were washed successively with small portions of dilute alkali and water to remove any adsorbed fatty acid, and the washings added to the solution. The soluble proteins and sugar were removed by the addition of 1 ml. of 25 per cent copper sulfate, followed by sufficient solid calcium hydroxide for alkalinity. After standing ½ hour, the mixture was centrifuged, and the clear supernatant liquid refluxed with mercuric sulfate according to the procedure of Van Slyke (4). The CO_2 liberated by the decomposition of the acetoacetic acid was swept, by a stream of nitrogen, into barium hydroxide solution, from which it was liberated by acid into an evacuated storage tube for determination of the C^{13} content. The precipitated acetone-mercury complex was transferred to a weighed sintered glass funnel, washed thoroughly, and dried at 110°. It was then oxidized by the wet combustion method of Van Slyke and Folch for determination of the C^{13} content.

The filtrate from the acetone precipitation was then treated with di-

chromate according to Van Slyke (4) fo
acid, and the acetone and CO_2 isolated
tions were made on the CO_2 evolved du
of acetone-mercury complex formed wa

Recovery of Residual Fatty Acids—T
ml. and the volatile acids determined
Duclaux distillations were carried out c
parison with the original octanoic acid.

Isotope Analysis—The C^{13} determina
mercial mass spectrometer manufacture
Corporation of Pasadena, California.

TABLE

*Distribution of C^{13} in Products of Incubation o
Liver Slices from*

Time, 2 hours; temperature, 37.5°.

	Experiment 1		
Tissue, gm.	1.23		
Volume of medium, ml.	30		
	mM	per cent C^{13}	
Octanoic acid utilized		1.59	0.
O_2 consumed	0.219		0.
CO_2 evolved in respiration		1.41	0.
Acetoacetic acid			
Acetone	0.0383	1.33	0.
Carboxyl		1.80	
Hydroxybutyric acid			
Acetone	0.0162		0.
Carboxyl			

been corrected for the 0.01 per cent of
contributing to the mass 45 peak as CO^{13}

Results

The results of four experiments in w
acid was incubated for 2 hours with liver
in Table I. The mean C^{13} content of the
acid is 1.32 ± 0.02 atom per cent, repres
over the normal complement of 1.01 per c
carboxyl group of the acetoacetic acid co
an excess of 0.78 per cent.

Location of C^{13} within Acetone Molecule—
C^{13} within the acetone the remaining D

according to the wet combustion
resultant CO_2 had a C^{13} conten
content for the carboxyl carbon of
in excellent agreement with the
tent of the original CO_2.
ices of 0.1 mm. thickness from rats
at 37.5° with 50 ml. of a 0.01 M
buffered Ringer's solution. The
ows: sodium octanoate 0.010 M,
10 M, $CaCl_2$ 0.001 M, KCl 0.002 M.
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ring the pH of the medium
and shaking continued an additional
of the liberated CO_2.
alkali-soaked filter paper in the
another Warburg vessel containing
swept out with a stream of CO_2-free
was liberated by acid tipped in
measured volumetrically. The
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determination of the C^{13} content.
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from the liver slices into a 50 ml.
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tant liquid refluxed with mercuric
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acid was swept, by a stream of
from which it was liberated by
determination of the C^{13} content.
was transferred to a weighed,
dried at 110°. It was then
d of Van Slyke and Folch for deter-
tion was then treated with di-

romate according to Van Slyke (4) for the oxidation of β-hydroxybutyric
d, and the acetone and CO_2 isolated as described above. C^{13} determina-
ns were made on the CO_2 evolved during the oxidation, but the amount
acetone-mercury complex formed was too low for C^{13} determinations.

Recovery of Residual Fatty Acids—The final filtrate was diluted to 200
ml. and the volatile acids determined by the method of Friedemann (5).
Dadaux distillations were carried out on the residual fatty acids for com-
parison with the original octanoic acid.

Isotope Analysis—The C^{13} determinations were carried out with a com-
mercial mass spectrometer manufactured by the Consolidated Engineering
Corporation of Pasadena, California. The C^{13} values given herein have

TABLE I

Distribution of C^{13} in Products of Incubation of 0.01 M Carboxyl-Labeled Octanoate with Liver Slices from Fasted Rats

Time, 2 hours; temperature, 37.5°.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Tissue, gm. / Volume of medium, ml.	1.25 / 30		1.25 / 30		2.50 / 60		2.50 / 60	
	mM	per cent C^{13}	mM	per cent C^{13}	mM	per cent C^{13}	mM	per cent C^{13}
Octanoic acid utilized		1.59	0.207	1.59	0.389	1.59	0.309	1.59
O_2 consumed	0.219		0.242		0.338		0.283	
CO_2 evolved in respiration		1.41	0.143	1.34	0.221	1.35	0.161	1.39
Acetoacetic acid	0.0383	1.33	0.0787	1.34	0.0579	1.30	0.0755	1.33
Acetone		1.80		1.89	0.0330	1.78	0.0659	1.85
Carboxyl								
Hydroxybutyric acid	0.0162		0.0143		0.0291		0.0376	
Acetone				1.22			0.1623	1.15
Carboxyl								

been corrected for the 0.04 per cent of O^{17} naturally present in oxygen contributing to the mass 45 peak as $CO^{16}O^{17}$.

Results

The results of four experiments in which carboxyl-labeled *n*-octanoic acid was incubated for 2 hours with liver slices from fasted rats are shown in Table I. The mean C^{13} content of the acetone moiety of the acetoacetic acid is 1.32 ± 0.02 atom per cent, representing an excess of 0.28 per cent over the normal complement of 1.04 per cent. The CO_2 derived from the carboxyl group of the acetoacetic acid contained 1.82 ± 0.03 per cent C^{13}, an excess of 0.78 per cent.

Location of C^{13} within Acetone Molecule—To ascertain the position of the C^{13} within the acetone the remaining Denigès mercury-acetone complex

from the four experiments was pooled, giving in all 40.2 mg., representin 2.01 mg. of acetone. It was dissolved in 2 ml. of 6 N HCl and the aceton distilled at room temperature under reduced pressure into a cooled mixture of 2 ml. of 15 N NaOH and 2 ml. of 0.1 N iodine solution. After warming to room temperature, the mixture was acidified. The iodoform was separated by centrifugation, washed thoroughly, and oxidized to CO_2 by the wet combustion procedure of Van Slyke and Folch. The CO_2 thus obtained had only the normal complement of C^{13}, 1.04 per cent. Since the iodoform in this procedure is derived from the methyl carbons, it is clear that the excess C^{13} of the acetone is exclusively in the carbonyl carbon. Inasmuch as the mean C^{13} content of the entire acetone molecule is 1.32, an excess of 0.28 per cent, the C^{13} excess of the carbonyl carbon is (3 × 0.28) or 0.84 per cent.

Respiratory CO_2—The mean value for the C^{13} content of the respiratory CO_2 was 1.37 ± 0.03 per cent, representing an excess of 0.33 per cent. Since the octanoic acid contained 1.59 per cent C^{13}, or an excess of 0.55 per cent, the average fraction of the respiratory CO_2 derived by complete oxidation of the substrate is 0.33/0.55 or 60 per cent of the total respiratory CO_2.

Disappearance of Octanoic Acid—In three of four experiments shown in Table I determinations were made of the amount of *n*-octanoic acid which disappeared during incubation. Of 0.207, 0.389, and 0.300 mM of octanoic acid utilized in these experiments only 0.0930, 0.0873, and 0.1131 mM respectively of total ketone bodies (as acetone) were recovered. Thus, of the octanoic acid disappearing, only 22, 11, and 18 per cent respectively can be accounted for as ketone bodies. Because of this surprising finding the method of recovery of the octanoic acid was carefully investigated. The residual acid was generally recovered after the successive steps of deproteinization, precipitation of acetone, and oxidation of β-hydroxybutyric acid. Preliminary experiments with known quantities of acetoacetic, β-hydroxybutyric, and *n*-octanoic acids of the same order as is dealt with in these experiments gave quantitative recoveries of each component. However, in order to rule out any possibility of loss of octanoic acid by any of these procedures, in Experiments 3 and 4 aliquots of the solution after incubation were acidified and distilled directly. The octanoic acid recovered by this procedure was not significantly greater than that obtained after deproteinization and ketone body isolation. The possibility that octanoic acid may be bound by the tissue was also investigated. A buffered 0.01 M solution of octanoic acid was divided into two equal parts, one of which was distilled directly, the other shaken with 1.25 gm. of liver slices and then distilled. The recovery of acid was quantitative in both

stances; hence there is no significant a
tissues.

Volatile Fatty Acids—The Duclaux con
and the volatile acids obtained by direc
compared in Table II. There is indicate
of a more volatile acid accompanying
butyric and hexanoic acids can be detect
tions, there is little likelihood they are
That the acid is possibly acetic acid is
Duclaux constants of the volatile acids
consisting of 90 per cent octanoic and 1
The constants are practically identical.

TABLE II
Duclaux Constants of

	12.5 per cen
n-Octanoic acid	88.0
Carboxyl-labeled *n*-octanoic acid	86.1
Volatile acids, Experiment 4	79.5
90% octanoic acid-10% acetic acid	79.0
Acetic acid	14.7

DISCUSSIO

Mechanism of Ketone Body Synthesis—T
(6, 7) assumes that successive β oxidation
the last 4 carbon atoms, in the form of buty
the ketone bodies. As originally conceive
the absence of acetic acid, which would b
result of β oxidation (8–10), and the forma
of more than 1 molecule of ketone body p
lized (11). As a result, several modificati
posed. To account for the high yields
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under reduced pressure into a cooled mix
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re is derived from the methyl carbons.
of the acetone is exclusively in the car-
ean C^{13} content of the entire acetone
er cent, the C^{13} excess of the carbonyl
cent.

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59 per cent C^{13}, or an excess of 0.55 per
respiratory CO_2 derived by complete
5 or 60 per cent of the total respiratory

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y of acid was quantitative in both

tances; hence there is no significant absorption of octanoic acid by the tissues.

Volatile Fatty Acids—The Duclaux constants for the initial octanoic acid and the voltaile acids obtained by direct distillation after incubation are compared in Table II. There is indicated the presence of a small amount of a more volatile acid accompanying the octanoic acid. Inasmuch as butyric and hexanoic acids can be detected by odor in very low concentrations, there is little likelihood they are present in significant amounts. That the acid is possibly acetic acid is indicated by comparison of the Duclaux constants of the volatile acids with those of a synthetic mixture consisting of 90 per cent octanoic and 10 per cent acetic acid (Table II). The constants are practically identical.

TABLE II

Duclaux Constants of Fatty Acids

	Total volume distilled				
	12.5 per cent	25.0 per cent	37.5 per cent	50.0 per cent	75.0 per cent
	Per cent of total acidity in distillate				
n-Octanoic acid	88.0	96.5	97.9	98.4	100.0
Carboxyl-labeled *n*-octanoic acid	86.1	97.0	98.2	98.8	100.0
Volatile acids, Experiment 4	79.5	91.0	94.3	96.1	100.0
90% octanoic acid-10% acetic acid	79.0	91.1	93.6	95.8	100.0
Acetic acid	14.7	30.1	46.4	63.9	100.0

DISCUSSION

Mechanism of Ketone Body Synthesis—The classical theory of β oxidation (6, 7) assumes that successive β oxidation along the fatty acid chain occurs, the last 4 carbon atoms, in the form of butyric acid, being oxidized directly to the ketone bodies. As originally conceived, this theory failed to account for the absence of acetic acid, which would be expected in large amounts as a result of β oxidation (8–10), and the formation, under certain circumstances, of more than 1 molecule of ketone body per molecule of fatty acid catabolized (11). As a result, several modifications of this theory have been proposed. To account for the high yields of ketone body by liver slices Jowett and Quastel (11) revived the hypothesis of Hurtley (8) that oxidation occurs at alternate carbon atoms throughout the fatty acid chain prior to splitting into units of 4 carbon atoms. Known as the theory of multiple alternate oxidation, it has been accepted widely (10–12).

Recently MacKay (13) has suggested that the ketone bodies may be formed by condensation of acetic acid (or some other 2-carbon intermediary)

resulting from β oxidation of the fatty acid. Although no experimental evidence was offered for this hypothesis, it is consistent with the known facts on which are based the earlier theories, and it explains certain findings which cannot be reconciled with the theory of multiple alternate oxidation; namely, (*a*) the formation of more ketone bodies from hexanoic than from butyric acid (12), and (*b*) the formation of ketone bodies from valeric acid, 3 carbon atoms of which are known to form glycogen (14, 15).

The essential correctness of the principle of β oxidation was conclusively demonstrated by Schoenheimer who found, with deuterium-containing fatty acids, the mutual interconversion *in vivo* of palmitic and stearic acids (16).

The presence of excess C^{13} in the acetone portion of acetoacetic acid formed by the breakdown of carboxyl-labeled *n*-octanoic acid is proof that a redistribution of the carbon atoms occurred during this process, a result which is inconsistent with either the classical theory of β oxidation or the multiple alternate oxidation hypothesis. Moreover, the equal distribution

TABLE III

Comparison of Observed Values of C^{13} Distribution in Acetoacetic Acid Predicted by Various Theories of Ketone Body Formation

	Atom per cent C^{13}, excess	
	Carbonyl	Carboxyl
Observed	0.84	0.83
β oxidation-condensation theory	1.10	1.10
Multiple alternate oxidation theory	0	2.20
Classical β oxidation theory	0	0

of the excess C^{13} between the carbonyl and carboxyl carbons of acetoacetic acid and the absence of excess C^{13} from the methyl and methylene carbons are consistent only with a mechanism involving the random coupling of identical 2-carbon units. The observed distribution of the excess C^{13} between the carbonyl and carboxyl carbons, compared with the values calculated for the three theories discussed above, is shown in Table III. The correspondence between the observed values and those predicted by the β oxidation-condensation mechanism is actually better than that indicated in Table III when it is recalled that in the absence of a substrate there is a spontaneous formation of ketone bodies sufficient to account for the differences noted.

The observations also rule out the mechanism for the formation of acetoacetic acid suggested by Krebs and Johnson (17), whereby it was presumed acetic and pyruvic acids condense to form acetopyruvic acid, which is then decarboxylated, according to the equation

$$CH_3COOH + CH_3COCOOH \rightarrow CH_3COCH_2COCOOH \rightarrow CH_3COCH_2COOH + CO_2$$

The results also preclude any possibility by a mechanism involving either α or ω hould be pointed out, however, that th liver slice experiments, may occur in the

Formation of Lower Fatty Acids—In β o theory, the assumption is made that a mol in this case hexanoic acid, is formed. If t remaining octanoic acid for the availabl involved, there should occur an accumul proceeds. The same considerations appl acetic. As a result, varying amounts of h would accumulate, depending on the relati formed. But these experiments gave no e hexanoic or butyric acid, and acetic acid, servations (10, 14), was present, if at all, with the amounts of octanoic acid catabol be offered for this phenomenon, though th experimental test: (*a*) the lower acids may olized much more rapidly than the origin acids may be so adsorbed to the surface of version to acetic acid occurs without disso fatty acids.

Nature of the 2-Carbon Intermediary—Alth fragment formed by β oxidation is not kn appears to be the most likely possibility, si pound known to form ketone bodies both (11, 13, 14). The experiments of Swends Xier (19) with carboxyl-labeled acetic acid

At first glance it is difficult to reconcile th tion of previous investigators that acetic a slower rate than the higher acids (11, 14). these *in vitro* studies the absorption of ac is slow and is the limiting factor in the ra ketone bodies. It is possible also that the acetic acid itself, but a more active derivati phosphate (20).

In view of the possible rôle of acetic a nthesis of ketone bodies, suggested by th Lehninger (21) has found an enzyme in mu catalyzes the reverse reaction, the breakdow

Other Possible Breakdown Pathways of Oc acid utilized in these experiments only abou

acid. Although no experiment
is, it is consistent with the known fac
ries, and it explains certain finding
ory of multiple alternate oxidation
ne bodies from hexanoic than from
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etone portion of acetoacetic acid
labeled *n*-octanoic acid is proof that a
occur during this process, a result
theory of β oxidation or the
is. Moreover, the equal distribution

III

ation in Acetoacetic Acid Predicted by
ne Body Formation

	Atom per cent C^{13}, excess	
	Carboxyl	Carboxyl
	0.84	0.83
	1.10	1.10
	0	2.20
	0	0

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$CH_3COCOOH + CH_3COCH_2COOH + CO_2$

the results also preclude any possibility of the formation of ketone bodies a mechanism involving either α or ω oxidation of fatty acid (18). It should be pointed out, however, that these mechanisms, ruled out in the liver slice experiments, may occur in the intact organism.

Formation of Lower Fatty Acids—In β oxidation according to the classical theory, the assumption is made that a molecule of the next lower homologue, in this case hexanoic acid, is formed. If the latter had to compete with the remaining octanoic acid for the available active surfaces of the enzymes involved, there should occur an accumulation of hexanoic acid as oxidation proceeds. The same considerations apply to the lower acids, butyric and acetic. As a result, varying amounts of hexanoic, butyric, and acetic acids would accumulate, depending on the relative rates at which each was transformed. But these experiments gave no evidence for the presence of either hexanoic or butyric acid, and acetic acid, in agreement with previous observations (10, 14), was present, if at all, in amounts small in comparison with the amounts of octanoic acid catabolized. Several explanations may be offered for this phenomenon, though they have not as yet been put to experimental test: (*a*) the lower acids may be oxidized or otherwise metabolized much more rapidly than the original 8-carbon acid; (*b*) the fatty acids may be so adsorbed to the surface of the enzyme that complete conversion to acetic acid occurs without dissociation of the intermediate lower fatty acids.

Nature of the 2-Carbon Intermediary—Although the nature of the 2-carbon fragment formed by β oxidation is not known with certainty, acetic acid appears to be the most likely possibility, since it is the only 2-carbon compound known to form ketone bodies both *in vitro* and in intact animals (11, 13, 14). The experiments of Swendseid, Barnes, Hemingway, and Nier (19) with carboxyl-labeled acetic acid are particularly convincing.

At first glance it is difficult to reconcile this conclusion with the observation of previous investigators that acetic acid forms ketone bodies at a lower rate than the higher acids (11, 14). It is possible, however, that in these *in vitro* studies the absorption of acetic acid through the cell wall is slow and is the limiting factor in the rate of its transformation to the ketone bodies. It is possible also that the 2-carbon intermediary is not acetic acid itself, but a more active derivative, such as, for example, acetyl phosphate (20).

In view of the possible rôle of acetic acid as an intermediate in the synthesis of ketone bodies, suggested by this work, it is noteworthy that Lehninger (21) has found an enzyme in muscle mince and bacteria which catalyzes the reverse reaction, the breakdown of acetoacetic to acetic acid.

Other Possible Breakdown Pathways of Octanoic Acid—Of the octanoic acid utilized in these experiments only about one-fifth or less can be ac-

counted for by the formation of ketone bodies (Table I). The question arises, therefore, as to other possible breakdown products. One possibility is complete oxidation to CO_2. Ordinarily it is impossible to know what proportion of the respiratory CO_2 comes from the substrate and how much represents oxidation of tissue constituents. With the aid of the carbon isotope, however, the CO_2 arising from the substrate may readily be calculated. In Experiment 4, for example, the fraction of the respiratory CO_2 which would arise from complete oxidation of the octanoic acid amounts to 62 per cent (see the computation under respiratory CO_2). Of the 0.161 mM of respiratory CO_2 obtained 0.161×0.62 or 0.0998 came from the substrate. Since each mole of octanoic acid yields 8 moles of CO_2 upon complete oxidation, the 0.0998 mM of CO_2 represents the complete oxidation of $0.0998/8 = 0.0125$ mM or only $(0.0125 \times 100)/0.309 = 4.0$ per cent of the total octanoic acid which disappeared. Similar calculations for Experiments 2 and 3 give values of 4.7 and 4.0 per cent respectively. Inasmuch as a portion of the respiratory CO_2 came from spontaneous decarboxylation of the acetoacetic acid, the portion arising by oxidation is probably lower than the calculated values. It is clear that only a small amount of octanoic acid was completely oxidized.

This conclusion is borne out by consideration of the oxygen consumed, which in each experiment was very much less than would be required if extensive oxidation of the octanoic acid had occurred. The results suggest that octanoic acid may be catabolized by some as yet unknown non-oxidative reaction, or its oxidation may be coupled with a reductive process. No products containing C^{13}, other than the ketone bodies, have as yet been isolated from these experiments, but the investigation is continuing.

SUMMARY

1. When *n*-octanoic acid, labeled by the incorporation of C^{13} in the carboxyl group, was incubated *in vitro* with liver slices from fasted rats, the resultant acetoacetic acid contained the excess C^{13} equally distributed between the carbonyl and carboxyl carbon atoms. The results offer unequivocal evidence that the ketone bodies are formed by condensation of a 2-carbon intermediary resulting from β oxidation of the fatty acid.

2. Although the nature of the 2-carbon intermediary formed by β oxidation is unknown as yet, the most probable substance is acetic acid.

3. Only 20 to 25 per cent of the octanoic acid which disappeared could be accounted for either by ketone body formation or by complete oxidation to CO_2.

We express our thanks to Professor H. C. Urey for kindly supplying us with the isotopic sodium cyanide used in this study. Our thanks are due

also to the Catalytic Develop
sylvania, for its cooperation,
spectrometer for the C^{13} analy

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ily it is impossible to know what pro-
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only (0.0125 × 100)/0.309 = 4.0
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CO_2 came from spontaneous de-
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oxidized.
ideration of the oxygen consumed,
less than would be required if
d occurred. The results suggest
some as yet unknown non-oxida-
d with a reductive process. No
tone bodies, have as yet been
investigation is continuing.

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Urey for kindly supplying us
ly. Our thanks are due
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Some Effects of High Lipid Diets on Intestinal Elimination
IV. Saturated Fatty Acids

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IN A PREVIOUS investigation made in this laboratory (1) some effects on intestinal elimination were noted when diets rich in glycerides of saturated fatty acids were fed to dogs. These effects included the following: (1) constipation, when diets containing large amounts of simple triglycerides of high molecular weight (tristearin and tripalmitin) were fed; (2) diarrhea, when small amounts of simple triglycerides of acids having twelve or less carbon atoms were fed. However, the addition to the diet of as much as 20 per cent trimyristin had no effect on intestinal elimination at all. Chemical analyses showed greater than normal amounts of lipids present in the feces when the glycerides fed melted above body temperature (tristearin, tripalmitin, trimyristin, trilaurin).

Several years ago, Bosworth, Bowditch and Giblin (2) noted that bottle-fed infants receiving diets too high in fat content were subject to vomiting and diarrhea. These investigators found increased quantities of calcium soaps present in the feces of such infants and attributed the gastro-intestinal behavior of the infants to the presence of these soaps. They also claimed that the fecal lipids would be further increased in such cases by neutral fats being occluded in the soaps.

Doell, Hallman and Reifman (3), who determined the rate of absorption of fatty acids in the rat found that butyric, caproic and caprylic acids fed as sodium salts were rapidly absorbed from the intestine, while capric acid (uncombined) was absorbed more slowly. Lauric acid became solidified and remained in the stomach so that it was absorbed very slowly. Lyman (4), feeding basic diets fortified with lard, ethyl palmitate, glyceryl tripalmitate, or palmitic acid to two dogs reported that the per cent of fatty acid (actually the ether extract) in the feces after ethyl palmitate or palmitic acid was much greater than after tripalmitin or lard feedings. He attributed the increase in ether extract to undigested ethyl palmitate in the first case, but the increase in palmitic acid when that lipid was fed could not be attributed to undigested material since no digestion was required. He, therefore, assumed that free fatty acids disturb the mechanism of digestion because they are irritating to the digestive tract, causing the food mass to be discharged more slowly from the stomach, but probably increasing peristalsis and the amount of feces.

Telfur (5), studying one normal infant and three with biliary atresia and obstructive jaundice, found that when bile is completely excluded from the gut, over 70 per cent of the dried feces are composed of fatty derivatives. Further, the non-absorption and persistence of excess fatty acids in the intestine are associated with modifications of the normal calcium and phosphorus excretion. Calcium is present in the feces in excess of normal, while phosphorus, released from calcium, is absorbed and eliminated in the urine. The extent of the variation is said to depend on the concentration of free fatty acids in the intestine.

While the preceding investigators believed that a relationship existed between dietary fat, fecal lipids and, in some cases, intestinal behavior, other investigators found no relation between ingested fats and fecal fat. Krakower (6), using eight fracture patients with good elimination habits as subjects, fed normal, low fat and special diets containing added corn oil (Mazola), or added coconut oil (Nucoa) for 4-day periods. Finding no correlation between the dietary fat and the total fat content of the feces or its composition as determined by the iodine number, he concluded that fecal lipids do not represent the residual food fat when moderate or larger amounts of fat are fed. Hill and Bloor (7), basing their conclusions on observations of four cats, decided that when moderate amounts of fat are fed, the fecal fat is independent of the diet, and that fecal fat ordinarily cannot be regarded as unabsorbed fat. They found the fecal lipids to consist principally of fatty acids and their salts (soaps) with smaller amounts of cholesterol and its derivatives and a little fat. They attributed the larger amounts of fat and fatty acids present in the feces after a high fat diet to unabsorbed food fat. Holmes and Kerr (8), using human subjects, also found the fecal fat to be different from that of the diet.

Because some of these investigators had attributed variations in either the behavior of the intestines or in the lipid content of the feces to the presence of unabsorbed fatty acids or calcium salts of fatty acids, it was decided to feed some of the fatty acids, the glycerides of which had been used in our previous experiment.

PROCEDURE

Four healthy male dogs*, the subjects in the previous experiment, were fed experimental diets. During this experiment, these dogs were kept indoors in sep-

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*Dog X, a thirty-pound part-spaniel; Dog C, a mixture of hound and collie, weighing about twenty-three pounds; and two fox terriers R and W, weighing seventeen and thirteen pounds, respectively.

Fat. Acid

arate cages, from which they were removed for periods of exercise. All were fed once a day and at the same time. Originally all of the dogs were given a diet of canned Pard* Dog Food, but when this article disappeared from the local markets, Dog X was fed dried food in order to conserve the scarce tinned Pard for the dogs used for the quantitative determinations. The smaller dogs, C, W, and R, were each fed the contents of a one-pound can of Pard each day. When fatty acids were to be introduced into the diet, the contents of a can of Pard (454 gm.) were thoroughly mixed with the calculated amount of acid in a mortar. The dog was then allowed to eat directly from the mortar in order to avoid loss of material through a transfer to another dish.

The large dog X, as in the previous investigation, was used principally to test the palatability of the special diets. When this dog refused to eat the food or became ill after eating it, the concentration of added fatty acid was diminished to a point where such effects were not observed before the diet was fed to the remainder of the dogs. If the dogs did not eat all of the special diets, the food was removed from the cage after half an hour. The dogs were not fed again till the next regular feeding time, 24 hours later.

When the special diets were fed, information about the intestinal behavior of the animal was obtained, as in the previous investigation, by direct observation of the animal and by chemical examination of the feces. The fecal material was collected for examination and analysed as quickly as possible after defecation in order to avoid changes in water content.

The special diets were fed for three consecutive days; the second day's ration being identified by a marker, so that the time required for the food to pass through the intestines could be easily noted.

Chemical analyses, run on the sample after mixing and grinding in a mortar until homogeneous, included moisture, total lipids, lipids soluble in neutral ether and soap fat. Iodine and acid numbers were determined on the three lipid fractions.

Moisture determinations were run simultaneously with lipid determinations in a Kaye extraction apparatus with mercury seals (9). Lipids soluble in neutral ether were extracted for twelve hours with neutral isopropyl ether, soap fats were then removed by extracting the residues for twelve hours with decinormal acid isopropyl ether. Total lipids were obtained by extracting the original samples with decinormal acid isopropyl ether for twenty-four hours and the values compared with the sum of the lipids soluble in neutral ether and soap fat for the same fractions.

All of the lipid fractions were purified by shaking at frequent intervals with petroleum ether, during a period of eight hours. The fractions were then filtered, the solvent distilled off and the residue dried in a vacuum dessicator over concentrated sulfuric acid. Iodine numbers (Wij's Method) and acid numbers were determined for the purified lipid fractions.

*Sufficient canned Pard Dog Food to complete these studies was furnished us by the Research Division of Swift and Company.

DISCUSSION AND RESULTS

Because the dogs had eaten a diet of Pard containing 20 per cent added tristearin in the previous investigation, an attempt was made to feed a diet of Pard to which 20 per cent stearic acid had been added. However, the dogs refused to eat such a diet and not until the stearic content had been reduced to five per cent would they eat their food at all. On the second day, all of the dogs were badly constipated, listless and had elevated temperatures. They refused to eat all of their rations, the amount left being inversely proportional to the size of the dog (W, 200 g.; R, 100 g.; C, 78 g.). On the third day, the symptoms of the previous day were still present, and the dogs refused to eat at all. Twenty-four hours later, the animals seemed to have recovered and ate their normal Pard diet.

Table I contains the analytical data for the fecal material in which the marker for the second day of the stearic acid diet appeared. For the purpose of comparison, data for the tristearin diet, as well as the normal diet, have been included.

Inspection of Table I shows that the addition of as little as five per cent stearic acid to the usual Pard diet of the dogs caused a reduction in the moisture content of the feces. The total lipid content of the feces of two of the dogs was less than when Pard diet alone was fed. The per cent of neutral ether extract increased in every case, and the per cent of soap fats diminished, just as was the case when the triglyceride of stearic acid was added to the diet. However, the total amount of soap present after the feeding of a diet containing 5 per cent added stearic acid was greater than after the diet with 20 per cent added tristearin.

The analytical constants of the fractions furnish little information about the composition of the lipids in the fractions. However, it can be said that the soaps isolated from the feces after feeding stearic acid do not consist primarily of stearic acid salts, since the acid numbers indicate material of much higher molecular weight.

The acid numbers ranging from 43.7 to 50.4 indicate molecular weights from 1283 to 1113, respectively, while the molecular weight of stearic acid is only 284. It, therefore, seems probable that, when stearic acid is fed, a great deal of the so-called soap extract in the feces does not consist of soaps of fatty acids at all.

The iodine numbers indicate a greater degree of unsaturation in the soap fraction when stearic acid was fed, than when either the Pard alone, or Pard with tristearin was fed.

Since there was no increase in calcium soaps in the feces even when stearic acid itself was added to the Pard diet, it seems reasonable to conclude that constipation resulting from a diet rich in tristearin or stearic acid is not caused by the presence of unabsorbed calcium soaps.

TABLE I

Analytical Data From Feces of Three Dogs Fed Tristearin and Stearic Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Fat by Extraction	Per Cent Lipids: Soap Fat by Diff.	Iodine Numbers: Total Lipids	Iodine Numbers: Neutral Ether Extract	Iodine Numbers: Soap Fat	Acid Numbers: Total Lipids	Acid Numbers: Neutral Ether Extract	Acid Numbers: Soap Fat
Pard	C	69.9	20.3	2.9	13.0	17.4	[illegible]	27.2	5.6	52.5	49.4	59.9
5% Stearic Acid		64.3	9.3	6.2	3.3	3.1	[illegible]	23.2	31.2	20.1	12.5	43.7
20% Tristearin		40.8	50.5	46.8	1.4	3.7	5.2	1.9	17.3	16.0	185.1	54.2
Pard	R	73.9	19.5	9.3	7.6	10.2	5.1	6.4	25.1	48.6	38.9	54.2
5% Stearic Acid		68.2	15.5	10.4	5.0	5.1	20.3	18.4	42.6	23.6	15.4	48.0
20% Tristearin		36.2	27.4	21.6	1.6	5.8	7.7	3.5	19.0	40.6	37.8	74.3
Pard	W	74.8	17.4	5.0	13.1	12.4	12.9	18.2	12.0	56.2	29.1	43.4
5% Stearic Acid		69.8	27.6	16.4	10.5	11.2	16.4	12.8	53.4	18.3	14.7	50.4
20% Tristearin		54.7	47.6	39.4	0.9	8.2	5.6	5.5	35.6	46.5	37.1	118.5

Because the dogs had all been made ill by the addition of five per cent stearic acid to the Pard diet, the experiment was not duplicated with palmitic acid. However, Dog C was fed a diet of Pard to which nine-three days all of the dogs ate a diet of Pard containing ten per cent additional lauric acid. During this period, the dogs were in good health and intestinal elimination was regular. The feces appeared normal in shape and consistency. It will be recalled that a Pard diet with 20 per cent added trilaurin in the previous

TABLE II

Analytical Data From Feces of Dog C Fed Tripalmitin and Palmitic Acid in Measured Quantities

Diet	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Extract	Per Cent Lipids: Soap Fat Difference	Iodine Numbers: Total Lipids	Iodine Numbers: Neutral Ether	Iodine Numbers: Soap Extract	Acid Numbers: Total Lipids	Acid Numbers: Neutral Extract	Acid Numbers: Soap Extract
Pard	69.9	20.3	2.9	18.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
Pard+0.9% Palmitic	70.6	18.4	12.0	4.7	6.4	36.2	36.7		159.7	194.2	154.1
Pard+20% Tripalmitin	63.3	56.9	42.7	9.8	14.2	9.3	7.4	8.4	96.6	93.8	201.2

tenths of a per cent palmitic acid was added. After this diet had been fed for three days, elimination was still normal. Analytical data for the feces included in Table II are not particularly informative.

The feeding of a meal of Pard to which lauric acid had been added was not difficult to accomplish. For investigation (I) caused diarrhea with feces only semi-solid in consistency.

Table III contains the analytical data for the fecal-material eliminated after the feedings of Pard with

TABLE III

Analytical Data From Feces of Dogs Fed Trilaurin and Lauric Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Fat by Extraction	Per Cent Lipids: Soap Fat by Difference	Iodine Number: Total Lipids	Iodine Number: Neutral Ether Extract	Iodine Number: Soap Fat	Acid Number: Total Lipids	Acid Number: Neutral Ether Extract	Acid Number: Soap Fat
Pard	C	69.2	20.3	2.9	18.0	17.4	-8.0	27.2	5.6	52.8	49.4	59.9
10% Lauric		48.9	12.3	7.4	5.0	4.9	15.1	12.7	16.9	18.0	15.0	42.4
20% Trilaurin		81.2	*54.6	42.0	12.6	—	—	14.4	26.8	—	125.8	187.9
Pard	R	73.9	19.5	9.3	7.6	10.2	5.1	6.4	25.1	48.6	38.9	54.2
10% Lauric		68.8	14.6	8.4	6.3	6.2	13.9	11.2	15.4	18.4	17.4	42.9
20% Trilaurin		78.4	13.0	9.3	1.5	3.7	23.8	22.8	48.1	241.2	199.7	214.8
Pard	W	74.8	17.4	5.0	13.1	12.4	12.9	18.2	12.0	56.2	29.1	43.4
10% Lauric		72.0	13.6	7.4	6.1	6.2	13.9	12.4	17.4	18.7	19.4	46.3
20% Trilaurin		79.7	18.8	15.1	2.0	3.7	17.6	18.3	36.0	235.7	200.8	244.1

*Total Fat by Calculation.

lauric acid. Data from the trilaurin diet are added for ease in making comparisons.

Inspection of Table III reveals a slight reduction in moisture content in the case of the two terriers and a marked reduction for Dog C. This is in decided contrast to the increase in moisture content after the feeding of the triglyceride of lauric acid.

The agreement between similar lipid fractions obtained from the feces of all three dogs on the lauric acid diets should be noted. In all cases, the percentage of total lipids is less than when Pard alone was fed. The extremely low acid numbers of all the soap fractions show that lauric acid (or its salts) is not the principal component of the fecal fat when the acid is fed. An acid number of 42.4 indicates a molecular weight of 1323; lauric acid has a molecular weight of 200. This, again, is a contrast to the soap fat of the feces after feeding the glyceride of lauric acid. The higher acid numbers (187.5-244.5) indicate lower molecular weights (200-229) not too different from that of lauric acid (200). This is the best agreement between the molecular weight of the fatty acid fed and the molecular weight of the fatty acid in the material recovered from the feces of any lipid diet that we fed, excepting that of tripalmitin.

The dogs were reluctant to eat Pard containing added caprylic acid but finally consumed all of a mixture with five per cent of the acid. This diet, fed for three consecutive days, proved mildly laxative and caused semi-fluid stools.

No particular difficulty was encountered with a Pard diet containing five per cent added caproic acid for all of the dogs ate the diet with apparent relish. Following this diet, the stools were profuse, soft and pasty.

Three of the dogs could not be persuaded to eat Pard to which butyric acid had been added. Dog X, when denied other food, finally ate a mixture of Pard containing two per cent butyric acid (about 1 gm. in a pound of Pard). Following this feeding, the stools were pasty. Only part of one feeding of a mixture of Pard with four per cent butyric acid was eaten by Dog X. This feeding gave rise to pasty stools with much fluid. No chemical analysis of the stools was attempted.

The analytical data for the feces collected after diets with added caprylic and caproic acids are included in Table IV. The lipid content was much the same after the feeding of caprylic acid or its triglyceride in the concentrations used. Caproic acid, fed in the same concentration as caprylic acid, resulted in much larger quantities of fecal lipids than caprylic acid; in two cases more than the normal amount. Since the total lipids and not the soaps increased after the caproic acid feedings, it was at first suspected that the material might consist of unabsorbed caproic acid. However, the low acid numbers of the fractions indicate that free caproic acid was not present in the feces to any extent.

The low acid numbers of the soap fat present in the feces after the diets with caproic or caprylic acids indicate once more that calcium soaps are not formed in appreciable amounts, after large amounts of free fatty acids are fed.

The acid numbers of all of the soaps obtained from the feces of the three dogs after feedings with acids are in remarkable agreement. The only exception is the case of palmitic acid where less than one per cent was fed. If this acid is omitted from the list, the highest acid number of the soap fats is 50.4, obtained when stearic acid was fed to the smallest dog; the lowest value is 12.3, obtained when caprylic acid was fed to the largest dog. The average value for all dogs and all acids is 45.4. These acid numbers correspond

TABLE IV

Analytical Data From Feces of Dogs Fed Tricaprylin, Tricaproin, Caprylic Acid and Caproic Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Extract	Per Cent Lipids: Soap Fat by Difference	Acid Numbers: Total Lipids	Acid Numbers: Neutral Ether Extract	Acid Numbers: Soap Extract
Pard	C	69.8	20.3	2.9	18.0	17.4	52.8	49.4	59.9
5% Caprylic Acid		70.2	4.1	2.3	1.8	1.8	16.0	14.0	42.5
4% Tricaprylin		77.8	4.2	1.7*	1.1	—	38.6	93.7	95.4
5% Caproic Acid		70.1	25.0	18.3	6.7	6.7	17.3	14.0	42.5
2% Tricaproin		78.3	6.8	6.2	1.9	2.6	74.6	60.6	77.4
1% Tricaproin		76.3	5.1	3.6	3.0	1.5	155.4	141.9	106.6
Pard	R	73.9	19.5	9.3	7.6	10.2	48.6	38.9	54.2
5% Caprylic Acid		71.5	7.0	4.5	2.2	2.5	17.4	12.2	47.0
4% Tricaprylin		75.3	6.7*	5.7	1.0	—	—	91.2	108.8
5% Caproic Acid		72.8	22.4	16.9	5.4	5.5	15.2	12.0	43.0
2% Tricaproin		82.7	4.2	3.1	1.2	1.1	98.7	92.6	116.7
Pard	W	74.8	17.4	5.0	13.1	12.4	36.2	29.1	43.4
5% Caprylic Acid		73.2	2.9	2.5	0.8	0.4	18.2	20.1	49.1
4% Tricaprylin		76.8	7.8	6.4	0.7	1.4	108.3	91.4	128.5
5% Caproic Acid		71.9	13.4	9.5	3.8	3.9	14.0	11.4	47.1
2% Tricaproin		80.3	5.5**	4.7	0.8	—	—	69.6	123.4

*Mechanical Loss.
**Total Fat by Calculation

to molecular weights of 1113, 1326 and 1235, respectively, a finding which strongly indicates that the materials did not contain any appreciable amount of fatty acid soaps. Samples of the so-called soap fats have, therefore, been preserved for further chemical analyses.

SUMMARY

1. Measured amounts of fatty acids mixed with a standard dog food (Pard) were fed to dogs to determine whether intestinal elimination would be affected by the added acid in the same manner as by the addition of the corresponding triglyceride.

2. Stearic, lauric, caprylic, and caproic acids were the acids mixed with the standard dog food and fed to all the dogs. One dog received feedings containing less than one per cent added palmitic acid; another ate a single feeding of Pard with added butyric acid.

3. Information concerning the intestinal behavior of the animals was obtained in two ways: (1) direct observation of the animal; and (2) chemical examination of the feces.

4. Chemical components of the feces for which analyses were made included moisture, total lipids, neutral ether extract and soap fat. Acid numbers were determined for all lipid fractions after all feedings; iodine numbers were determined after stearic and lauric acid feedings.

5. Constipation was produced by 5 per cent added stearic acid.

6. The neutral ether extract increased for every animal after the 5 per cent stearic acid feedings but the so-called soap fat was less than the normal value in every case.

7. The addition of 10 per cent lauric acid to the normal diet did not affect the normal rate of elimination of the dogs.

8. The feeding of diets containing caprylic, caproic or butyric acid produced laxative effects in all cases. The intensity of the action varied inversely with the number of carbon atoms just as in the case of the triglycerides of the corresponding acids. The moisture content of the feces did not vary greatly from the normal values when caprylic or caproic acid was added to the diet. No quantitative estimations were made after feeding butyric acid.

9. The acid numbers of all of the fatty acids from the soap extracts, with the exception of the one palmitic acid experiment, ranged from 42.3 to 50.4, with an average value of 45.4. These values correspond to molecular weights of 1326, 1113 and 1236, respectively.

CONCLUSIONS

The effect of a high fat diet on intestinal elimination depends at least partially on the component acids of the fats fed. Dogs fed pure saturated fatty acids behaved in approximately the same manner as when fed the corresponding pure triglycerides. Diarrhea resulted from the feeding of diets containing caprylic, caproic or butyric acids, or the glycerides of these acids. Constipation followed feedings of stearic acid or tristearin. Although trilaurin produced diarrhea, elimination was normal after feeding lauric acid.

These effects cannot be attributed to the presence in the feces of large amounts of free fatty acids or of soaps of the fatty acids, because the low acid numbers of the total lipid and neutral ether extracts exclude the possibility of large amounts of free acid. The low acid numbers of the soap fractions rule out the possibility of large amounts of soaps of fatty acids.

The low acid numbers of all of the fractions indicate that neither the fatty acid as fed nor its soaps are present in the feces to any extent. These low acid numbers can only be explained by assuming that small amounts of free fatty acids are mixed with large amounts of non-titratable material or by a very large molecule having an acid radicle.

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Enrichment of depot fat with odd and even numbered medium chain fatty acids[1]

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ZURIER, ROBERT B., ROBERT G. CAMPBELL, SAMI A. HASHIM, AND THEODORE B. VAN ITALLIE. *Enrichment of depot fat with odd and even numbered medium chain fatty acids.* Am. J. Physiol. 212(2): 291–294. 1967.—Medium chain fatty acids shorter than laurate normally are absent in adipose tissue lipids. When these acids are present in dietary fats, they are transported via portal vein to liver where they are extensively metabolized. Transport of medium chain fatty acids into systemic circulation was achieved in rats with portacaval shunts. Enrichment of adipose tissue with medium chain fatty acids was effected by altering route of their transport to circumvent liver. Control animals and those with anastomoses were fed diets containing medium chain triglycerides of which the fatty acid constituents were C9 or C8 and C10 for periods up to 3 months. Fatty acid patterns by gas liquid chromatography were determined from samples of omental adipose tissue prior to and following portacaval shunting. Striking increases in proportions of C9 or C8 and C10 fatty acids of adipose tissue were noted in animals with portacaval shunts. By enriching depot fat with odd chain fatty acids such as pelargonate, it is possible to store appreciable quantities of fatty acids of which the terminal three carbon units are potentially glucogenic.

adipose tissue (omentum); portacaval anastomosis; pelargonate, caprylate; fatty acid transport

PLASMA FREE FATTY ACIDS (FFA) represent the transport form of lipid originating from adipose tissue and are readily available for oxidation or incorporation into lipid ester moieties of lipoprotein. Information concerning the transport and fate of FFA has been derived largely from studies of the behavior of long chain fatty acids. In contrast, medium chain fatty acids which differ markedly from the long chain fatty acids with respect to solubility and interaction with proteins usually are not constituents of the FFA transport system in the intact animal.

Alteration in the long chain fatty acid content of rat adipose tissue has been induced experimentally (7, 19, 20, 25, 28). Since ingested medium chain fats are transported as FFA in portal venous blood (14, 15, 22) directly to the liver where they are oxidized nearly completely (21), it is difficult to enrich the fat stores with medium chain fatty acids by feeding medium chain triglyceride. In the present study, an alteration of the route of transport of medium chain fatty acids to circumvent the liver was achieved by feeding odd or even numbered medium chain triglycerides to rats with portacaval shunts. In this way, medium chain FFA were transported directly into the systemic circulation. Thus, it was possible to induce striking increases in proportions of medium chain fatty acids stored in adipose tissue.

METHODS

Twenty-six male Sprague-Dawley rats weighing 250–300 g had been maintained on a nutritionally adequate stock diet deriving 26% of calories from fat (soy bean and corn oils: 50% linoleate), 54% from carbohydrate (wheat and corn meals), and 20% from protein (casein). The animals were separated into four groups depending on the sequence of portacaval anastomoses and medium chain triglyceride feeding. In *group 1* (10 rats), an end-to-side portacaval anastomosis was performed on each animal. At operation a sample of omental fat was obtained. In a control group of rats of comparable weight (*group 2;* 8 rats) a sham operation was performed at which time omental fat also was obtained. Both groups of rats were placed in individual cages, allowed to convalesce, and were fed an experimental regimen consisting of a basic fat-free diet (Nutritional Biochemical Corp., Cleveland, Ohio), supplemented with an odd numbered medium chain (C9) triglyceride (tripelargonin). The diet, as modified, was adequate in vitamins and minerals and derived 17% of calories from protein (casein), 53% from carbohydrate (sucrose), and 30% from fat (tripelargonin).

The animals in *groups 3* and *4* also were subjected to an initial sham operation, at which time samples of omen-

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TABLE 1. *Fatty acid patterns (percent of total) of adipose tissue of four groups of rats prior to and during feeding of tripelargonin and even numbered medium chain triglyceride.*

Fatty Acid	All Groups (26)	Group 1 (10)	Group 2 (8)	Group 3 (2)		Group 4 (6)	
	Prior to feeding of exptl. diets	1 Month after portacaval anastomoses and tripelargonin	1 Month after sham operation and tripelargonin	1 Month after sham operation and tripel argonin	1 Month after portacaval anastomoses and 2 months after tripelargonin	1 Month after sham operation and even numbered medium chain triglyceride	1 Month after portacaval anastomoses and 3 months after even numbered medium chain triglyceride
C6:0						0.5±0.3	5.4±1.2
C8:0		12.4±3.9	1.9±1.4	3.9	15.8		
C9:0				0.2	0.2	1.4±1.0	4.6±1.5
C10:0				0.2	0.3	0.6±0.5	1.1±0.2
C12:0				1.3	2.5	3.1±0.6	2.4±0.6
C14:0	1.7±0.2	1.4±0.6	1.7±0.6	31.0	28.9	32.6±1.7	28.5±5.3
C16:0	25.1±4.0	28.0±5.7	29.7±5.8	7.6	15.8	12.3±1.8	13.4±1.2
C16:1	8.1±3.1	11.1±4.7	10.3±2.8	3.5	2.7	5.6±1.7	2.6±0.4
C18:0	3.1±0.8	3.1±0.8	2.7±0.4	38.4	26.2	38.1±3.3	37.0±4.3
C18:1	30.7±4.2	29.5±4.3	36.9±6.1	12.3	2.6	4.8±2.0	5.0±2.0
C18:2	28.6±5.2	11.9±7.3	17.0±12.1				
Other	1.3±0.8	1.2±0.9	0.8±0.8				

Data are means ± SD; numbers of observations are noted in parentheses.

tal fat were obtained. The animals in *group 3* (2 rats) received the same experimental diet (tripelargonin-supplemented) as *groups 1* and *2*, but after 29 days on the diet they underwent portacaval anastomoses and omental biopsies. Then, they continued on the same diet for an additional 29 days, following which samples of omental fat were obtained again. The animals in *group 4* (6 rats) were subjected to the same operative procedures and in the same sequence as those in *group 3*. The rats in *group 4* consumed an experimental diet similar in all respects to those fed the other groups except that the fat source was even numbered medium chain triglyceride isocalorically substituted for tripelargonin. Thus, each animal in *groups 3* and *4* served as its own control. Animals in all groups remained healthy and gained weight during the feeding of the experimental diets. The gains in body weight of animals that had undergone sham operations were comparable to those of the animals which had received portacaval anastomoses and these animals were included in the experimental groups. Fatty acid patterns of all the omental fat samples were determined by gas liquid chromatography following transmethylation of the extracted lipid with sodium methoxide.

RESULTS

The fatty acid pattern of adipose tissue of the four groups of rats is shown in the second column of Table 1. It is apparent that the adipose tissue of all groups of rats prior to feeding of the experimental diets contained no fatty acid shorter than myristate (C14:0). Linoleate (C18:2) accounted for an appreciable proportion of the long chain fatty acids in the fat depot of the rats, reflecting their previous intake of a linoleate-rich diet. In *group 1* rats in whom tripelargonin feeding was begun following portacaval shunting, the pelargonate content 1 month after portacaval anastomosis was 12.4% of the fatty acids of the adipose tissue. In contrast, the group of sham-operated rats (*group 2*) had less than 2% pelargonate in their adipose tissue. In animals of *groups 3* and *4* that were fed odd and even numbered medium chain triglyceride, respectively, prior to and following portacaval anastomoses, the adipose tissue was enriched slightly with medium chain fatty acids prior to the shunting operation, and strikingly after operation.

The change in the long chain fatty acid patterns of adipose tissue in response to feeding of the experimental diets in the four groups of animals also is shown in Table 1. It is apparent that the feeding for 1 month of these regimens to sham-operated animals was associated with a drop in the proportion of linoleate and a concurrent rise in oleate of adipose tissue lipid. The enrichment of the adipose tissue with odd and even numbered fatty acids after feeding the corresponding triglycerides to animals with anastomoses occurred principally at the expense of linoleate in *groups 1*, *2*, and *3*. There was no appreciable change in the depot fat proportions of linoleate and oleate in animals of *group 4* during even numbered medium chain triglyceride feeding following portacaval anastomosis. However, slight decreases in the saturated fatty acid series were noted during enrichment of adipose tissue with C8:0 and C10:0 fatty acids.

DISCUSSION

It has been possible to enrich adipose tissue glycerides with appreciable amounts of odd and even numbered medium chain fatty acids by shunting the portal venous blood of rats fed the parent triglycerides into the systemic circulation. Thus, if they are made to bypass the liver, medium chain fatty acids, though they are presumably unesterified, readily enter adipose tissue. Also, in this way, the adipose tissue can be enriched with odd num-

d fatty acids of which the terminal three carbon units are potentially glucogenic.

In contrast, studies of the distribution of albumin-bound ^{14}C-labeled palmitate in rats 15 min after intravenous injection have disclosed a general uptake of the label by liver, muscle, and other organs. It is of particular note that adipose tissue had no detectable radioactivity (6). This was the case in the fed as well as the postabsorptive animal. On the other hand, injected chylomicrons were taken up primarily by adipose tissue in the fed animal. It appears that re-esterification of long chain free fatty acids and their incorporation into lipoprotein may be necessary before such fatty acids can be stored in adipose tissue (5, 8, 10).

The fatty acids derived from orally administered medium chain triglyceride are transported via the portal vein in the unesterified form (14, 15, 22), while those derived from long chain triglycerides are transported in lymph as lipid ester moieties of chylomicrons (4, 17). The medium chain fatty acids are extensively oxidized by the liver and feeding medium chain fats does not lead to any appreciable deposition in adipose tissue lipids of the intact animal (11, 24, 27). In the present study, the small amounts of medium chain fatty acids found in the adipose tissue of the sham-operated animals may be related to a slight degree of chylous transport of medium chain acids in triglyceride form (23). There was more decanoate (10:0) than caprylate (C8:0) in the sham-operated rats in group 4 despite the fact that decanoate was only 20% of the triglyceride that was fed. This suggests a greater degree of chylous transport for decanoate than for caprylate. Also, more decanoate than caprylate may have been transported from the liver to the adipose tissue as a constituent of plasma lipoprotein.

Medium chain triglycerides are hydrolyzed in the intestine faster than long chain triglycerides (3, 9, 13) and are absorbed under circumstances which adversely affect long chain glyceride absorption (16, 18, 29). This may be due to the fact that the products of hydrolysis of medium chain triglycerides are more soluble than those derived from long chain triglycerides (7), permitting absorption directly through the gut mucosa and into the portal capillary bed without re-esterification. The same mechanism may apply for entry of medium chain fatty acids into the adipose tissue cell. Indeed, adipose tissue displays cellular processes characteristic of pinocytosis (21), and it has been suggested that pinocytosis may be involved in the absorption of fat by the intestine (26).

Another mechanism may be considered. As chain lengths of fatty acids decrease, the fatty acid-albumin bond is weakened (1, 12). It is conceivable that when a medium chain FFA, such as pelargonate, comes in contact with the membrane of the adipose tissue cell, this bond is easily broken, facilitating entry of the fatty acid to the cell. In any event, the greater affinity which serum albumin has for long chain fatty acids than for medium chain fatty acids is a pronounced physicochemical difference which, in part, may help to account for the different behavior of these fatty acids in biological systems.

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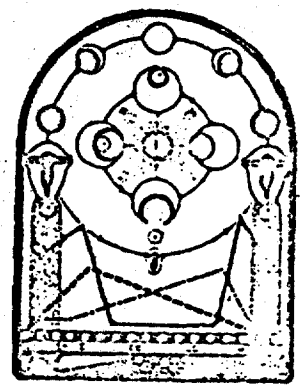

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Nutritional Evaluation of Medium-Chain Triglycerides in the Rat

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Abstract

Nutritional evaluation of a medium-chain triglyceride (MCT) preparation, containing about 75% octanoic acid and 25% decanoic acid, was carried out in short- and long-term experiments in rats. A casein diet containing 19.6% MCT and 2.5% safflower oil, the latter to supply essential fatty acids, was compared with similar diets containing conventional dietary fats. Data obtained in a 47-week study showed that the MCT diet supported normal growth and development. At autopsy, carcass protein and ash levels, vital organ weights and composition were similar to those in rats fed conventional fats. Histological study showed that intestinal and liver sections were normal after 47 weeks on the MCT-containing diet. In general, rats fed MCT had slightly lower growth rates and caloric efficiency values, less carcass fat and smaller epididymal fat pads than animals fed conventional dietary fats. Little C_8 and C_{10} were found in depot fat. The MCT diet also supported normal reproducton, as indicated by litter size and number. During lactation the volume of milk secreted by the rats receiving the MCT diet was smaller and contained a lower level of fat than that secreted by the rats receiving an oleo oil diet, resulting in slower gains in weight in the MCT group. After weaning, growth of the rats fed MCT compared favorably with that attained by the animals on the diet containing oleo oil.

Introduction

In recent years a number of papers have appeared which indicate that the medium-chain triglycerides (MCT) are of particular value in the nutritional management of individuals who are unable to effectively absorb dietary fat. MCT is of special importance in malabsorption syndromes which arise from a deficiency of pancreatic lipase (1), bile salts (2), or intestinal absorptive area (3). Because of ease of absorption (4–8), the calories in MCT are available to maintain positive caloric balance and body weight in individuals who are unable effectively to absorb conventional dietary fats.

Only a few papers have appeared which demonstrate the nutritional value of the medium-chain triglycerides. Kaunitz et al. (9) demonstrated that male rats which were fed diets containing MCT for a year weighed significantly less than animals fed a lard diet and had higher caloric requirements to maintain weight than animals fed a lard or a fat-free diet. Kaunitz et al. also concluded that linoleic acid requirements are less with MCT than with long-chain triglycerides (C_{12-18}) (10). In the past few years the authors' laboratory has been engaged in a series of studies designed to obtain more information on the nutritional and biochemical aspects of MCT so that this unique triglyceride might be used for special dietary needs. Although much of this work has not yet been published, two long-term studies with MCT, a 47-week growth study beginning with weanling rats and a reproduction and lactation study, are now reported.

Methods

In the long-term growth study, groups of 15 male and 15 female rats of the Wistar strain were fed diets which differed only in the source of dietary fat. Throughout the 47-week study the animals were housed in individual screen-bottom cages in an air-conditioned animal room. The diets contained sufficient fat to supply 40% of the calories from MCT, oleo oil, butter fat, coconut oil, corn oil, and safflower oil. Safflower oil (2.5%) was added to the diets containing MCT, oleo oil, butter fat, and coconut oil to insure adequacy of the essential fatty acids in all diets. The diets also supplied 38% of the calories from carbohydrate, 22% of the calories from protein, and adequate amounts of vitamins and minerals (Table I).

Records of food intake were kept for the calculation of calorie efficiency and net absorption of fat, protein, and calcium. At intervals during the study, feces were collected daily, pooled in weekly samples, and analyzed for fat (11), total nitrogen (Kjeldahl method), and for calcium (12). At specified intervals, blood was obtained from the tail vein and analyzed for cholesterol by method of Abell et al. (13).

At the termination of these studies the rats were killed, and the weights of the livers, kidneys, spleen, heart, adrenals, femurs, testes, and epididymal fat pads were determined. Liver and intestine were examined histologically; liver and carcass were analyzed for fat by method of Folch et al. (14) and protein by Kjeldahl. Phospholipid levels in the liver were also determined (15). The fat content of the fat pads was determined (11), and fatty acids were measured by gas chromatography (16) after methylation (17).

In the reproduction and lactation study, groups of young adult male and female rats of the McCollum-Wisconsin Strain were fed the MCT and oleo oil diets, as above, and a third group was given a low-fat diet containing 2.5% safflower oil (Table I, Diet 7). After three weeks on these diets the animals were mated. The females were housed in a separate room, and each

TABLE I
Composition of Diets

	Diet 1–6 40% Fat calories	Diet 7 Low fat
	%	%
Fat[a]	21.0	2.5
Casein (ANRC 91.8% protein)	26.2	26.2
Amidex[b]	44.5	63.0
Nonnutritive fiber	4.0	4.0
Mineral mixture[c]	4.0	4.0
Vitamin mixture[d]	0.35	0.35

[a] Diets 1–4 contained mainly MCT, oleo oil, butterfat, and coconut oil respectively, with 2.5% safflower oil added to insure adequate essential fatty acids. (The level of the fat in the MCT diet was increased slightly since MCT provides only 8.3 cal/g.) Diets 5 and 6 contained corn oil and safflower oil respectively.

[b] Partially hydrolyzed corn starch, Corn Products Company, New York.

[c] J. H. Jones and C. Foster (J. Nutr. 24, 245, 1942) with 10 ppm F added as NaF.

[d] H. P. Sarett and L. P. Snipper (J. Nutr. 52, 525, 1954). Ascorbic acid omitted. In addition, 0.015 g Dicum Percomorphum and 0.005 g dl-α-tocopherol acetate were added per 100 g diet.

TABLE II

Weight Gains and Caloric Efficiency Values for Male and Female Rats Fed Different Dietary Fats for 47 Weeks

Dietary fat	Males: 4-Week caloric efficiency	Males: Weight gain after 4 wks	Males: Weight gain after 8 wks	Males: Weight gain after 47 wks	Females: 4-Week caloric efficiency	Females: Weight gain after 4 wks	Females: Weight gain after 8 wks	Females: Weight gain after 47 wks
	g gain/1,000 cal.	g	g	g	g gain/1,000 cal.	g	g	g
MCT[a]	115 ± 10	181 ± 21	332 ± 44	637 ± 94	101 ± 5	139 ± 12	209 ± 21	375 ± 83
Oleo oil[a]	119 ± 6	200 ± 22[b]	354 ± 39	668 ± 115	99 ± 5	137 ± 10	208 ± 20	403 ± 76
Butter fat[a]	118 ± 6	201 ± 15[b]	356 ± 32	669 ± 97	101 ± 8	146 ± 16	223 ± 29	417 ± 81
Coconut oil[a]	121 ± 5	197 ± 14[b]	353 ± 25	715 ± 102[b]	98 ± 16	142 ± 13	215 ± 18	432 ± 76
Corn oil	122 ± 9	196 ± 19[b]	353 ± 38	630 ± 62	100 ± 5	140 ± 11	220 ± 19	414 ± 39
Safflower oil	119 ± 7	188 ± 17	345 ± 37	674 ± 71	103 ± 6	139 ± 15	212 ± 21	400 ± 64

[a] Plus 2.5% safflower oil.
[b] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE III

Weights of Epididymal Fat Pads and Carcass Composition of Rats Fed Different Dietary Fats for 47 Weeks

Dietary fat	Epididymal fat pads		Carcass Composition[b]			
			Weight	Fat	Protein	Ash
	g	% body wt.	g	%	%	%
MCT[a]	14.6	2.2	580 ± 80	22.1 ± 5.9	19.4 ± 1.6	3.0 ± 0.3
Oleo oil[a]	18.7	2.6	607 ± 91	22.4 ± 4.9	20.1 ± 2.0	3.1 ± 0.5
Butter fat[a]	19.1	2.7	612 ± 83	24.7 ± 5.5	19.4 ± 1.9	3.0 ± 0.6
Coconut oil[a]	23.2[c]	3.1[c]	644 ± 89[c]	26.7 ± 6.1[c]	18.2 ± 1.7	2.8 ± 0.5
Corn oil	16.7	2.5	572 ± 51	23.7 ± 3.3	19.0 ± 0.8	3.1 ± 0.4
Safflower oil	17.9	2.5	610 ± 61	22.6 ± 4.0	19.8 ± 1.1	3.0 ± 0.3

[a] Plus 2.5% safflower oil.
[b] Does not include liver, heart, epididymal fat pads or gastrointestinal tract.
[c] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE IV

Fatty Acid Content of Dietary and Epididymal Fat in Rats Fed Various Dietary Fats for 47 Weeks

	Fatty acids, %											
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:4}$	Other
Dietary Fat												
MCT[a]	51.0	35.0	2.0		0.9			1.4	2.0			0.7
Oleo oil[a]				2.9	22.1	4.8	13.4	43.2	12.5			1.1
Butter fat[a]	1.9	3.3	2.9	8.1	22.8	3.8	10.5	23.3	18.3			10.1
Coconut oil[a]	8.1	7.2	36.8	17.2	10.0		2.4	7.2	11.0			0.1
Corn oil					13.4		1.4	26.2	57.9			1.2
Safflower oil					6.7		1.9	10.0	80.9	0.2		0.4
Epididymal Fat												
MCT[a]	0.4	4.9	1.5	2.2	21.9	8.3	2.3	30.8	25.1	0.7	0.4	1.5
Oleo oil[a]				1.4	16.2	4.0	5.0	59.6	10.6	1.0	0.2	1.8
Butter fat[a]			1.1	5.1	22.4	6.1	4.2	45.2	11.7	1.9	0.1	2.2
Coconut oil[a]		0.7	20.3	12.9	15.7	4.6	1.1	26.2	17.4	0.4	0.1	0.6
Corn oil				0.3	11.9	1.2	1.6	33.0	50.2	0.6	0.6	0.6
Safflower oil				0.4	9.4	1.0	1.8	17.4	68.0	0.4	0.9	0.7

[a] Plus 2.5% safflower oil.

was kept in a large plastic cage with wood shavings during pregnancy and lactation. After weaning, selected young from each litter were housed in the screen-bottom cages and raised to maturity for further reproduction and lactation studies in succeeding generations.

In order to estimate the volume of milk secreted during lactation, the mother was removed from her litter for a six-hour period every third day. Milk secretion was estimated by the increase in weight of the pups measured just prior to and immediately after a one-hour nursing period after the return of the mother. In a separate reproduction study, milk was obtained from the lactating rats receiving the MCT and oleo oil diets, by the method of Cox and Mueller (18), for proximate analysis and determination of fatty acid content.

Growth Studies

Weight gains of the rats receiving the MCT, oleo oil, butterfat, coconut oil, corn oil, and safflower oil diets after 4, 8, and 47 weeks and caloric efficiency values for the first four weeks are shown in Table II. Weight gains with MCT were only slightly less than with other fats. After four weeks the male rats fed the MCT diet gained 181 g compared to gains of 200 g with oleo oil, 201 g with butter fat, 197 g with coconut oil, 196 g with corn oil, and 188 g with safflower oil. Four-week caloric efficiency values were also similar with all dietary fats, varying between 115 and 122 g gained per 1,000 calories. At the end of 47 weeks, weight gains with MCT, in both males and females, were moderately lower than those with most of the other dietary fats. There was a significant difference only between the values with the MCT and coconut oil diets in the male rats.

Mortality was not markedly different in the groups receiving the various fats during the study. During the 47 weeks an average of 2.5 rats died per group of 15 males and 1.7 per group of 15 females; in the groups receiving MCT, 3 males and 2 females died during the study.

Organ weights of the liver, kidneys, spleen, heart, adrenals, femurs, and testes were determined at the end of the study and were similar in all groups; data are not given. Histological examination of the liver and intestine also show no marked differences among the groups receiving the different dietary fats.

Weights of the epididymal fat pads were found to be affected by the type of dietary fat, as shown in

Table III. In the group receiving the MCT diet, the fat pads were 2.2% of the body weight, as compared with values of 2.5 to 3.1% in the groups receiving the other dietary fats. These data correlate fairly well with the level of fat in the carcass. Animals consuming MCT had the lowest level of carcass fat. Levels of protein and ash in the carcass were similar with all dietary fats.

Fatty acid composition of depot fat was influenced by dietary fat and varied widely among the groups. In each group, epididymal fat composition was quite similar to that of carcass fat; data on fatty acids in epididymal fat are shown in Table IV along with composition of dietary fats. The high levels of C_{12} in coconut oil, and $C_{18:2}$ in corn oil and safflower oil were reflected in the high levels of these fatty acids in the epididymal fat. However low levels of C_8 and C_{10}, 0.4 and 4.9% respectively were found in the fat pads of the rats fed MCT although these fatty acids comprised about 85% of the dietary fat. High levels of palmitic acid (21.9%) and of oleic acid (30.8%) were in the fat pads of the rats fed MCT although only traces of these fatty acids were in the diet. Examination of the data suggest that fatty acids such as C_{12}, C_{14}, and $C_{18:2}$ are apparently utilized to a great extent for direct incorporation into tissue fat; C_8 and C_{10} are rapidly metabolized to smaller units, and little of these is directly incorporated into tissue fat; C_{16} and $C_{18:1}$ are the main fatty acids synthesized.

It is interesting that the level of $C_{18:2}$ in the epididymal fat of the MCT and coconut oil-fed groups were 2.8 and 1.6 times the levels in the diets respectively whereas, with the other diets, the epididymal fat contained 0.8 to 0.9 as much $C_{18:2}$ as the dietary fats. This may be related to the presence of the shorter-chain fatty acids in MCT and coconut oil; these are mostly metabolized, thereby indirectly increasing the relative amount of $C_{18:2}$ in the fat made available to the animal.

Total plasma cholesterol levels measured at intervals during the study (Table V) were lower in male rats receiving the MCT diet than in those receiving the other dietary fats. This relationship was not apparent in female rats. In both sexes the highest levels were found in the groups receiving the coconut oil diet. At the end of the study, blood samples for more complete cholesterol analyses were obtained by cardiac puncture after the animals were fasted for 18 hours. Under these conditions the lowest values were found in the animals on the corn oil and safflower oil diets; with MCT the values were higher. The differences in findings between the earlier values and the terminal values may have been attributable in part to differences in conditions under which the blood samples were obtained or may reflect changes in age of the animals or in length of time on the diets.

Total liver lipids and cholesterol levels were lower in both male and female on the MCT diet than in those receiving the other dietary fats (Table VI). Phospholipid levels were similar on all diets and were apparently not affected by the type of dietary fat in both males and females. The difference between total lipids and the sum of phospholipids and cholesterol presumably represents the triglyceride fraction. This

TABLE V

Plasma Cholesterol Levels in Rats Fed Various Dietary Fats for 47 Weeks

	Weeks							
	7	14	21	35	47			
	Plasma cholesterol							
	Total				Total	Free	Esterified	Esterified
	mg/100 ml				mg/100 ml			%
Males								
MCT[a]	84 ± 9	85 ± 13	92 ± 15	99 ± 15	100 ± 28	27 ± 7	73 ± 23	73 ± 5
Oleo oil[a]	105 ± 18[b]	110 ± 20[b]	116 ± 26[b]	117 ± 33[b]	86 ± 26	22 ± 8	64 ± 20	74 ± 5
Butter fat[a]	110 ± 16[b]	108 ± 15[b]	123 ± 19[b]	126 ± 29[b]	92 ± 28	22 ± 7	72 ± 21	76 ± 3
Coconut oil[a]	112 ± 9[b]	115 ± 13[b]	128 ± 13[b]	135 ± 12[b]	113 ± 28	25 ± 7	89 ± 27	78 ± 3[b]
Corn oil	110 ± 21[b]	104 ± 24[b]	118 ± 25[b]	115 ± 19[b]	81 ± 17	15 ± 4[b]	66 ± 18	82 ± 1[b]
Safflower oil	100 ± 17	97 ± 17[b]	109 ± 23	105 ± 18	82 ± 23	15 ± 7[b]	67 ± 18	82 ± 3[b]
Females								
MCT[a]	109 ± 13	107 ± 11	119 ± 19	126 ± 21	124 ± 26	33 ± 9	92 ± 19	74 ± 4
Oleo oil[a]	106 ± 15	104 ± 13	107 ± 13	116 ± 25	102 ± 36	26 ± 9	73 ± 27	74 ± 2
Butter fat[a]	110 ± 13	108 ± 9	125 ± 19	122 ± 23	126 ± 15	33 ± 6	92 ± 13	74 ± 4
Coconut oil[a]	124 ± 20[b]	125 ± 19[b]	142 ± 25[b]	148 ± 28[b]	125 ± 31	28 ± 8	97 ± 24	79 ± 3[b]
Corn oil	96 ± 14[b]	96 ± 11[b]	103 ± 19[b]	112 ± 17	93 ± 37[b]	17 ± 9[b]	76 ± 29	82 ± 3[b]
Safflower oil	88 ± 13[b]	83 ± 11[b]	101 ± 12[b]	107 ± 13[b]	90 ± 16[b]	17 ± 9[b]	74 ± 13[b]	82 ± 4[b]

[a] Plus 2.5% safflower oil.
[b] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE VI

Liver Lipid Composition in Rats Fed Various Dietary Fats for 47 Weeks

Dietary fat	Liver weight	Total lipid		Phospholipids	Cholesterol
Males	g	mg	%	mg/g	mg/g
MCT[a]	14.3 ± 1.9	889 ± 236	6.2 ± 1.2	30 ± 2	2.5 ± 0.4
Oleo oil[a]	14.5 ± 2.9	991 ± 340	6.7 ± 1.0	24 ± 3[b]	3.1 ± 0.4[b]
Butter fat[a]	13.9 ± 1.6	1040 ± 375	7.4 ± 2.2	24 ± 2[b]	3.0 ± 0.5[b]
Coconut oil[a]	16.1 ± 3.6	1317 ± 435[b]	7.8 ± 1.0[b]	24 ± 3[b]	3.8 ± 0.3[b]
Corn oil	13.8 ± 1.9	1073 ± 243	7.7 ± 0.9[b]	22 ± 3[b]	4.0 ± 0.6[b]
Safflower oil	15.7 ± 3.9	1429 ± 1052[b]	9.1 ± 3.6[b]	23 ± 3[b]	4.7 ± 1.3[b]
Females					
MCT[a]	9.8 ± 1.7	705 ± 282	7.1 ± 1.8	24 ± 3	2.2 ± 0.3
Oleo oil[a]	10.4 ± 1.8	937 ± 277[b]	8.9 ± 1.7[b]	24 ± 3	2.5 ± 0.3
Butter fat[a]	10.9 ± 1.8	857 ± 238	7.8 ± 1.2	24 ± 2	2.4 ± 0.2
Coconut oil[a]	11.1 ± 2.3	988 ± 266[b]	8.9 ± 1.5[b]	25 ± 2	2.4 ± 0.3
Corn oil	11.5 ± 2.0	894 ± 182[b]	7.9 ± 1.7	24 ± 3	2.8 ± 0.4[b]
Safflower oil	10.1 ± 1.8	907 ± 282	8.9 ± 1.7[b]	24 ± 2	3.1 ± 0.3[b]

[a] Plus 2.5% safflower oil.
[b] $P < 0.05$ compared with MCT group.
All values are shown with standard deviations.

TABLE VII
Net Absorption of Dietary Fat, Protein, and Calcium from Diets with Different Dietary Fats

	Percentage of Dietary Intake				
			Calcium absorption		
	Fat absorption 35 wks	Protein absorption 35 wks	3 wks	10 wks	21, 35, and 47 wks (Average)
	%	%	%		
Males					
MCT[a]	97	94	70	41	20
Oleo oil[a]	78	93	68	41	16
Butter fat[a]	89	94	71	45	17
Coconut oil[a]	96	94	73	44	20
Corn oil	95	93	67	39	20
Safflower oil	95	91	74	43	21
Females					
MCT[a]	98	91	74	35	26
Oleo oil[a]	85	93	73	25	22
Butter fat[a]	94	91	74	36	17
Coconut oil[a]	96	92	78	42	21
Corn oil	97	91	72	40	15
Safflower oil	97	94	70	38	22

[a] Plus 2.5% safflower oil.
100 g of diet supplied about 21 g fat, 23.6 g protein from casein, and 454 mg of calcium (447 mg from $CaCO_3$ and 6.8 mg from casein).

fraction was also lower in the MCT groups than in those on the other diets.

Net absorption of fat, protein, and calcium during various periods of the study are shown in Table VII. The values were calculated from dietary intakes and fecal excretion with no correction for endogenous losses. Values were obtained on group-pooled samples and do not permit statistical comparisons. The net absorption of MCT was higher than that of the other dietary fats; there was little difference in protein absorption. Calcium absorption was apparently not markedly affected by the type of fat in the diet. However retention of calcium decreased with age; about 70% during the third week, 30 to 40% in the 10th week, and about 20% later in the study.

Reproduction and Lactation Study

The F_0 generation (young adult male and female rats of the McCollum-Wisconsin Strain) were maintained on the MCT diet, the oleo oil diet, and the low-fat diet for three weeks prior to mating. The average number of pups per litter and birth weights of the F_1 generation are shown in the upper half of Table VIII. Findings were similar on all three diets. Weight gains during weaning were lower on the low-fat diet than on the MCT or oleo oil diet; at 21 days male rats on the MCT diet averaged 45 g and those on the oleo oil diet 47 g whereas those on the low fat diet weighed only 39 g. Mortality during the lactation period was 6, 7, and 2% on the three diets respectively.

After weaning the F_1 rats were raised on the same diets fed to their mothers. Weights at 49 and 105 days of age were again lowest in the group receiving the low-fat diet. This difference in weight gain is related in part to food intake since caloric efficiencies were similar on all three diets.

At 12 weeks of age each group of F_1 rats was divided into three subgroups; one subgroup was continued on the same diet whereas the two other subgroups were switched to the diets containing one of the other two fats, thus providing nine experimental groups, as shown in the lower portion of Table VIII. After three weeks the F_1 females were mated; data on their pups, the F_2 generation, are summarized in Table VIII. The number of pups per litter and birth weights were also similar for all the subgroups. However weight gains of the pups during lactation were related to the fat in the diet of the mothers during this period. In general, the highest weights at weaning (21 days) were found in the groups on the oleo oil diet except for the slightly low value in that group which had previously received the low-fat diet. Intermediate weaning weights were found in the groups receiving the MCT diet, and the lowest weaning weights were found in the groups receiving the low-fat diet.

Mortality was relatively high in two of the groups receiving MCT, the one previously on MCT (22%) and that previously on the low-fat diet (20%), but not in the group previously on the oleo oil diet (6%). Mortality was 7% or less in the other six subgroups. Subsequent growth of these animals showed no marked differences, as shown by weights of males at 63 days. Determination of the amount of milk secreted by the mothers of each subgroup suggested that this factor may have affected weight gain and mortality.

The increase in weight of the litters during a timed lactation period was used as an index of milk secretion; these values are summarized in Table IX. During these seven periods the F_0 mothers secreted on the average about 40, 41, and 43 g of milk on the MCT, oleo oil, and low-fat diets respectively, showing no difference because of diet. There was much greater variation in milk secretion by the F_1 mothers. However milk secretion was quite low in those animals which had received the MCT diet for two generations.

The composition of the milk obtained from F_0 mothers receiving the MCT and oleo oil diets was determined in a previous study. Data in Table X suggest that the level of fat in the milk of the animals receiving MCT is slightly lower than that in the milk of the rats receiving oleo oil. Although 85% of the

TABLE VIII
Birth Weight and Body Weights of Rats Born and Nursed by Mothers Receiving MCT, Oleo Oil, and Low-Fat Diets

Dietary fat[a]	Pups per litter	Day: Birth	Day: 6	Day: 12	Day: 18	Day: 21	Day: 49	Day: 63	Day: 105
		Male and Female				Male			
		Weight, g per rat				Weight, g per rat			
F_1 Generation									
MCT	9.0	6.4	13	21	34	45	151		309
Oleo oil	9.1	6.1	14	24	34	47	156		326
Low fat	9.6	6.4	13	22	29	39	165		286
F_2 Generation									
MCT / MCT	9.2	6.5	12	23	35	45		261	
Oleo oil / MCT	7.0	6.7	12	23	36	45		242	
Low fat / MCT	9.4	6.6	12	23	36	43		243	
MCT / Oleo oil	9.4	6.8	13	25	39	49		249	
Oleo oil / Oleo oil	9.2	6.3	12	24	39	47		244	
Low fat / Oleo oil	10.5	6.0	11	23	35	43		246	
MCT / Low fat	10.8	6.2	11	21	31	36		244	
Oleo oil / Low fat	8.8	6.4	12	25	36	39		245	
Low fat / Low fat	9.3	6.5	12	23	32	38		243	

[a] All diets contained 2.5% safflower oil.

TABLE IX

Milk Secreted by F_0 and F_1 Generation Lactating Rats Receiving MCT, Oleo Oil, and Low-Fat Diets[a]

Dietary fat[b]	Day 3	6	9	12	15	18	21	Total
	g milk							
F_0 Generation								
MCT	3.3	4.7	5.0	5.5	7.1	7.3	7.3	40.2
Oleo oil	4.3	6.5	4.5	5.3	6.5	6.0	6.0	41.2
Low fat	4.5	5.6	6.3	6.2	5.4	6.7	8.4	43.1
F_1 Generation								
MCT / MCT	1.7	0.8	4.0	2.3	4.0	5.2	6.3	24.3
Oleo oil / MCT	1.3	2.0	2.8	4.3	6.2	9.9	8.8	34.4
Low fat / MCT	2.4	3.9	5.0	5.0	6.7	8.7	5.5	37.2
MCT / Oleo oil	2.2	3.6	6.2	7.0	6.6	10.2	9.2	45.0
Oleo oil / Oleo oil	1.5	2.2	4.8	5.8	6.3	10.8	9.8	41.2
Low fat / Oleo oil	2.2	4.8	7.5	8.7	8.3	8.3	10.0	49.8
MCT / Low fat	1.8	6.5	5.3	4.0	7.5	11.2	10.0	46.3
Oleo oil / Low fat	2.0	4.2	5.2	4.4	7.6	9.6	8.0	41.0
Low fat / Low fat	1.6	3.9	4.4	5.3	6.6	7.7	9.1	38.6

[a] Milk secretion was estimated as the increase in weight of each litter during a one-hour lactation period; the mother was removed from the litter for six hours beforehand.
[b] All diets contained 2.5% safflower oil.

TABLE X

Fatty Acid Composition of Milk Obtained from Lactating Rats Receiving MCT- or Oleo Oil-Containing Diets

Dietary fat[b]	Milk fat	Fatty acid composition of milk fat[a] — Fatty acid No. 8	10	12	14	16	18	16:1	18:1	18:2	18:3	20:4	Other
	%												
MCT	8.2	6.5	16.8	10.3	11.5	29.9	4.7	0.9	11.7	6.3	0.3	0.3	0.4
Oleo oil	9.8	2.2	5.8	4.4	6.6	20.8	9.4	2.4	36.7	8.8	0.9	0.4	2.8

[a] Milk was obtained on 10th day of lactation.
[b] Diets also contained 2.5% safflower oil.

dietary fatty acids were C_8 and C_{10} in the MCT group, these constituted only 24% of the milk fat fatty acids. In contrast, the fatty acids in the milk secreted by the oleo oil group were similar to those contained in the dietary fat. Fatty acid composition of these milks show appreciably higher levels of saturated fatty acids C_8 to C_{16} in the milk of the MCT group and markedly more $C_{16:1}$, C_{18}, and $C_{18:1}$ in the milk of the rats fed oleo oil. It is apparent that the rats fed MCT were required to synthesize a large portion of the fatty acids secreted in the milk fat.

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