SODIUM SALTS OF FATTY ACIDS #81 Vol. I

SODIUM SALTS OF FATTY ACIDS #81 Vol. II
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VOLUME 1

GRAS MONOGRAPH SERIES
SODIUM SALTS OF FATTY ACIDS

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PREFACE

This monograph, while entitled Sodium Salts of Fatty Acids, deals only with the sodium salts of oleic and palmitic acids.

Because of the difference in effects noted for the saturated palmitate compound and the unsaturated oleate compound, the two are treated in two separate sections in this monograph, A single summary, bibliography, and set of reference documents are presented, however.

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SUMMARY

OLEIC ACID, SODIUM SALT

Description and Specifications

Sodium oleate exists as a white to yellowish powder with a slight tallow-like odor. It is soluble in about 10 parts alcohol. Commercial specifications (141) limit free fatty acid to 2.5% maximum, 2.0% maximum moisture and ash (as Na_2CO_3) to 17% minimum and 18% maximum.

Acute Toxicity

Oro and Wretlind (097) found an LD_{50} value for oleic acid of 230 $\pm$ 18 mg/kg injected intravenously to mice.

No mortality was observed by Day *et al*. (027) when oleic acid was infused intravenously to dogs at 10 mg/kg over a five minute period.

Short-Term Studies

Lecoq *et al*. (077) found that rats ingesting 20-22% sodium oleate showed increased excitability in the neuromuscular system, shortened nervous chronaxia, and increased muscle chronaxia.

Kinosita *et al*. (066) observed morphological changes in the rat liver following one intraperitoneal injection of sodium oleate. Although the cells regained normal structure, the liver remained plump, unaccompanied by cirrhotic changes.

Blood extracted from dogs surviving an intravenous infusion of sodium oleate by Day *et al*. (027) showed a

large decrease in platelet count which was inconsistent with the only slight change observed in serum prothrombin time.

Special Studies

Nakahara (090) showed that an intraperitoneal injection of sodium oleate to mice increased their resistance to subsequent cancer transplant. In the same study, sodium oleate was found to have no significant increase in resistance to cancer already *in situ*. Nakahara suggested the possibility that resistance resulted from an increased lymphoid tissue activity following sodium oleate injection.

Rabbits fed sodium oleate simultaneously with cholesterol showed intensification of experimental atherosclerosis. Novikova (095) suggested that this is due to increased absorption of exogenous cholesterol and decreased excretion of endogenous cholesterol in the presence of oleic acid, as well as to increased deposition in plaques of cholesterol esters along with oleic acid.

Oro *et al*. (097) injected an oleic acid emulsion into cats. They found that at a dosage of 50 mg/kg, there was a hypertensive effect on the systemic circulation and a hypotensive effect on the pulmonary artery. The authors noted that the extent of the effects was dependent upon the emulsifier employed.

Absorption and Excretion

Bergstrom *et al*. (008) administered labeled oleic acid via gastric intubation to rats. The authors found that after 24 hours, a mean of 78.2% of the activity was absorbed. They also showed the insignificance of phospholipid as a carrier of the absorbed oleic acid. About 16% was expired as CO_2 after 24 hours.

Effects on Enzymes and Other Parameters

In an *in vitro* test, Surgala *et al*. (121) found oleate and other unsaturated fatty acids to be fibrinolytic.

Kerr *et al*. (065) demonstrated that an oleate-lecithin sol produced transient platelet aggregation.

Yamamoto (142) studied the effect of sodium oleate on isolated rat mitochondria. He found that structural changes (shrinkage) of the mitochondria and pyridine nucleotide oxidation resulted from 0.1 μmole sodium oleate per mg mitochondrial protein. This result could be reversed by adding bovine serum albumin. Yamamoto felt that the mechanism of action of the sodium oleate could not be successfully explained.

A decrease in activity of the enzyme diastase when reacted with sodium oleate was observed by Cameron (018).

Reyner (106) found that both the surface tension of whole blood and the sedimentation rate of erythrocytes were decreased by addition of sodium oleate.

Greisman (043) demonstrated the hemolytic properties of sodium oleate and described the saturation levels and

conditions for rates of hemolysis. He also found no change in hemolytic activity by adding non-specific enzyme inhibitors. The author suggested that hemolytic substances released following oleate hemolysis were responsible for the capability of sodium oleate for inducing a decelerating hemolytic chain reaction.

Rochman et al. (108) gave intravenous and intraperitoneal injections of sodium oleate to rats which resulted in a marked decrease in gastric secretory function. The authors noted that this inhibition did not depend upon contact of the oleate with intestinal mucosa. Kowalewski (069) obtained similar results in rats which were histamine stimulated as well as in non-stimulated rats.

Crespin et al. (023) noted acute elevations of plasma insulin and significant increases in pancreaticoduodenal vein insulin levels in dogs receiving infusions of sodium oleate.

Drug Interaction

Lecoq et al. (077) observed a reversal of toxic effects in rats ingesting sodium oleate when nicotinamide, pantothenic acid or pyridoxine was given orally.

Consumer Exposure

Although the sodium salts of fatty acids do not naturally occur in food, they may be added as binders, emulsifiers

or anti-caking agents. No information regarding production levels, annual poundage or possible daily intake is available.

SUMMARY

PALMITIC ACID, SODIUM SALT

Description and Specifications

Sodium palmitate occurs as a white or faintly yellow crystalline solid or as a white or yellowish-white powder. It is soluble in both water and alcohol. The Joint Expert Committee on Food Additives (060) specifies that sodium palmitate should contain not more than 3 ppm arsenic nor more than 10 ppm heavy metals (as Pb).

Acute Toxicity

Oro and Wretlind (097) found an LD_{50} value for palmitic acid of 57 $\pm$ 34 mg/kg injected intravenously into mice.

Cucuianu, *et al*. (024) observed mortality in six of 16 dogs following an intravenous infusion of 6.8 mg/kg sodium palmitate.

Day, *et al*. (027) obtained 33% mortality rate in dogs infused intravenously with 10 mg/kg sodium palmitate.

Short-Term Studied

Herting, *et al*. (055) found rapidly produced lipogranuloma in weanling rats fed palmitic acid. This reaction could be reversed by replacing the palmitic acid with corn oil.

Cucuianu, *et al*. (024) examined blood from dogs receiving daily infusions of sodium palmitate. Platelet counts varied normally while platelet adhesiveness decreased when tested 24 hours after infusions. Fibrinogen levels and euglobulin lysis times were also studied.

Day, et al. (027) found massive venous and arterial thrombi in dogs infused with sodium palmitate. Blood extracted from surviving dogs showed significant shortening of recalcification time.

Special Studies

Nakahara (090) showed that sodium palmitate exhibited no immunologic effect upon subsequent cancer implantation.

Absorption and Distribution

Bloom, et al. (011) showed absorption of 81-95% of intragastric intubated labeled palmitic acid. The majority of the activity was recovered in the lymph. In a later study (010), the same authors found that 96% of the labeled palmitic acid in the lymph was in non-phospholipid form.

Herting, et al. (055) found absorption of 58% and 58.9% of palmitic acid fed to weanling rats over a one week period.

Metabolism and Excretion

Baker, et al. (003) calculated the rates of plasma free fatty acid exchange, esterification and oxidation, hepatic triglyceride formation and non-triglyceride formation in fasted rats injected with labeled palmitic acid.

Effects on Enzymes and other Parameters

Surgala, et al. (121) found sodium palmitate and other saturated fatty acid salts to be less fibrinolytic than the unsaturated fatty acid salts.

Kerr, et al. (065) demonstrated that a palmitate-

lecithin sol produced transient platelet aggregation.

Goodman (042) found evidence that the initial steps in plasma unesterified fatty acid metabolism involved transfer of free fatty acids from serum albumin to binding sites on the erythrocyte.

Ehrenstein and Corey (031) demonstrated the increased effect of testosterone on the accessory sex organs of the male rat by injecting palmitic acid or sodium bipalmitate.

Drug Interaction

Hoshino, *et al*. (057) showed that sodium palmitate increased the absorption of leucomycin from rat mucous membrane *in vitro*.

Consumer Exposure

Although the sodium salts of fatty acids do not naturally occur in food, they may be added as binders, emulsifiers or anti-caking agents (060). No information regarding production levels, annual poundage or possible daily intake was available. The acceptable daily intake of sodium palmitate has not been limited, provided the contribution of cations does not exceed the normal body load (061).

CHEMICAL INFORMATION

I. Nomenclature

A. Common names: Sodium oleate, oleic acid, sodium salt

B. Chemical names: *cis*-9-octadecenoic acid, sodium salt

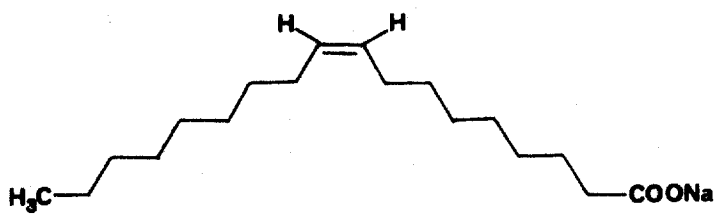
C. Trade names: Eunatrol, Olate Flakes

D. Chemical Abstracts Service Unique Registry Number: 143191

II. Empirical Formula

$C_{18}H_{33}O_2Na$

III. Structural Formula



IV. Molecular Weight: 304.45

V. Specifications

A. The WITCO Chemical Corporation lists the following specifications for sodium oleate, technical and sodium oleate EA Food Grade (141): (Specifications are identical for the two grades, however, food grade sodium oleate is made from food grade oleic acid.)

	Min.		Max.
Free Fatty Acid	--		2.5
Moisture	--		2.0
Ash (as Na_2CO_3)	17.0		18.0
Free Alkalinity	--	none	--
Fineness-- 100 Mesh, %	50		
20 Mesh, %	100		
Color	Cream to light tan		

VI. Description

A. General Characteristics:

Sodium oleate exists as a white to yellowish powder with a slight tallow-like odor.

B. Physical properties:

Sodium oleate is soluble in about 10 parts water and 20 parts alcohol. Due to hydrolysis, it is alkaline in aqueous solutions, but not in alcoholic solutions.

C. Stability in containers:

Although unsaturated fatty acids are usually less stable than the saturated fatty acids, oleate stability is not a problem when it is maintained at room temperature in a closed container under normal conditions of humidity.

VII. Analytical Methods

Although, in general, the separation and identification of individual fatty acids is a difficult matter, common separation techniques are applicable, such as thin-layer chromatography, silica gel and gas-liquid chromatography.

The following methods of analysis are quantitative for salts (generally sodium) of long chain fatty acids (palmitic, oleic, stearic, etc.):

1. The Official Methods of Analysis of the Association of Official Agricultural Chemists (AOAC), 10th Edition (002) has given official status to a method for quantifying soaps (salts of fatty acids).

The sample, in petroleum ether, is extracted several times with 50% alcohol. The alcohol layers are combined and evaporated. The residue is dissolved in H_2O made alkaline and extracted with ether. Ether layers are combined and evaporated. The residue is weighed as fatty acids. From the weight of the fatty acids, the percent soap in the sample is calculated as sodium or potassium oleate.

Note is made that error will result if the apparent molecular weight of fatty acids differs appreciably from that of oleic acid.

2. The Standard Methods of Chemical Analysis (138) presents a method in which 75-100 g of sample are ashed and the ash added to 50 ml of cold, boiled distilled water. The solution is titrated with 0.02 N acid to a methyl orange endpoint.

$$\% \text{ Soap as sodium oleate} = \frac{\text{ml of } 0.02\ \underline{N} \text{ acid} \times 0.608}{\text{Wt. of sample}}$$

3. The Standard Methods of Chemical Analysis (138) also presents a titrimetric method for quantitative analysis of soaps. To a 100 ml solution of acetone containing 2% water and 0.5 ml of 0.1% bromophenol blue in 95% alcohol is added 0.01 N hydrochloric acid to the first appearance of a permanent yellow color. A test tube containing 40 g of sample and 1 ml of water is warmed and shaken. 50 ml of the neutralized acetone solution is added (again with warming and shaking) and the mixture is then allowed to stand until layers separate. Green or blue in the upper layer indicates the presence of soap.

The upper layer is titrated with 0.01 N hydrochloric acid to the reappearance of the yellow color.

$$\% \text{ Soap as sodium oleate} = \frac{\text{ml of } 0.01\ \underline{N} \text{ acid} \times 0.304}{\text{Wt. of sample}}$$

This method is reported to be satisfactory for determining soap in oil within the range of 10-500 ppm.

VIII. Occurrence

Oleic and palmitic acids do not normally occur as their sodium salts, but rather as triglyceride esters in natural fats. The sodium (or potassium)

salts of these acids, or soaps as they are commonly referred to, are the product of saponification of animal and vegetable fats and oils; that is, alkaline hydrolysis of the fatty acid esters usually with sodium (or potassium) hydroxide.

Oleic acid, which is commonly obtained from olive oil, generally constitutes more than 30% of all fats and is found present in all natural fats or phosphatides.

In the animal organism, the fatty acids are stored and transported as neutral fats (triglycerides) or phospholipids. There may be a small amount of free fatty acid in the blood which is present as soap (098) due to the alkaline pH and the presence of cations such as sodium and potassium.

BIOLOGICAL DATA

I. Acute Toxicity

A. Mice

Oro and Wretlind (097) injected a 2% emulsion (for composition, see Table 1) of oleic acid intravenously into the tail vein of mice of at least 6 groups of 10 animals each (weighing 13-34 g). An LD_{50} value of 230 ± 18 mg/kg BW was found for oleic acid. (See results of a similar experiment, Palmitic acid, sodium salt, Biological Data, Section I, A.)

Table 1

Composition of 2% Emulsion of Oleic Acid (097)

Oleic acid	2 g
Cottonseed oil	10 g
Phosphatides	0.4 g
Sodium cholate	0.1 g
Glycerol monostearate	0.5 g
5% glucose solution to	100 ml

(Emulsion adjusted to pH 7.3 with N NaOH)

B. Dogs

Day et al. (027) studied the effects of a single rapid infusion of sodium oleate on dogs. Intravenous infusions over a five minute period of various fatty acids in doses of 10 mg/kg BW (as a 0.1% suspension of their sodium salts) were performed on 195 male mongrel dogs weighing an average of 10 kg, judged healthy by the authors. No

Table 2

Acute Toxicity
(by substance or constituent thereof)

Substance	Animal (Species)	Sex & No. (M or F)	Route (p.o.,i.v.,s.c., i.p.,i.m., other)	Dosage mg/kg body wt.	Measurement (LD50, ED50 or other	Ref. Bibliogr. No.
Oleic acid	mice	at least 6 groups, 10 animals per group	i.v.	230 ± 18	LD_{50}	Oro and Wretlind (097)
Sodium oleate	dogs	M, 29	i.v. (rapid infusion)	10	0% mortality	Day *et al.* (027)

mortality was observed in the dogs receiving sodium oleate. When sodium oleate was infused as a mixture with sodium palmitate or sodium stearate, mortality rates of 10% and 71% respectively were observed (See Table 3).

Table 3

Acute Toxicity of Sodium Oleate
and other Fatty Acids (027)

Substance (sodium salts)	No. of Dogs	Sex	Route	Dosage mg/kg BW	% Mortality
Oleate	29	M	i.v.	10	0
Linoleate	20	M	i.v.	10	0
Stearate	36	M	i.v.	10	25
Palmitate	45	M	i.v.	10	33
Oleate-Palmitate	20	M	i.v.	10	10
Oleate-Stearate	28	M	i.v.	10	71
Saline (control)	17	M	i.v.	10	0

Thrombi were found in only one necropsied dog treated with oleate. The authors concluded that when infused intravenously in dogs over a five minute period in doses of 10 mg/kg BW as a 0.1% suspension of their sodium salts, the unsaturated fatty acids (oleate) were not thrombogenic as compared to saturated fatty acids and suggested that at least in the case of the palmitate-oleate mixture, the oleate produced partial protection against the lethal effect of palmitic acid. (See results of similar experiment, Palmitic acid, sodium salt, Biological Data, Section I, B, 2.)

II. Short-Term Toxicity

A. Rats

1. Chauchard *et al.* (019) found that feeding sodium oleate daily (20-22%) to rats (number, sex, weight, and age not given) increased the excitability of the neuromuscular system, shortened nervous chronaxia and increased muscle chronaxia. The same results were obtained by daily subcutaneous injections of sodium oleate (no dosages given).

2. Kinosita *et al.* (066) observed the effect of one intraperitoneal injection of 10 mg sodium oleate in 1 ml water on rat liver (number, age, and weight not given). The liver became plump with rounded edges "a few days" following the injection. Examination of the liver cells under the electron microscope showed unstained patches of irregularly-shaped cytoplasm, shortage of ribosomes, and alteration of the endoplasmic reticulum. The liver cells, though regaining their usual structure eventually, retained marked dilation of their central veins and hyperplasia. The liver remained plump, unaccompanied by cirrhotic changes. Females were more sensitive to sodium oleate than males.

B. Dogs

Day *et al.* (027) examined male mongrel dogs which survived a single, rapid intravenously infused dose of sodium oleate (10 mg/kg BW as a 0.1% suspension over a five minute period). Dogs surviving similar infusions of other fatty acid salts were also examined. Using the Wessler technique, immediate necropsy of three dogs surviving the sodium oleate infusion revealed thrombi in one dog. However, the study indicated that, in general, unsaturated fatty acids in contrast to saturated ones were not thrombogenic.

In vitro coagulation tests were performed on blood extracted from the surviving dogs. Results of these tests are shown in Table 4. Sodium oleate produced some of the largest drops in platelet count (partially dilutional). This could not be correlated with the observation that it also produced the smallest reduction in serum prothrombin time.

In studying lipids in the extracted blood, no significant changes were encountered in the sodium oleate infused dogs nor in those dogs infused with other fatty acids. (See Table 5.)

The authors noted that the coagulation studies apparently provided *in vitro* evidence that the *in vivo* effect in those animals receiving unsaturated fatty acids (oleate) differed from those receiving saturated fatty acids. (For similar experiment, Palmitic acid, sodium salt, see Biological Data, Section II, B, 2.)

III. Long-Term Toxicity

No information available.

IV. Special Studies

A. Mice

Nakahara (090) tested the effects of oleic acid and sodium oleate on the resistance of mice to transplanted cancer. The sodium oleate was a 1% solution in distilled water, and the oleic acid was a 1% emulsion. (Emulsion was prepared either with distilled water and sufficient sodium hydroxide to stabilize it, or from the sodium oleate solution adding hydrochloric acid. No difference in results between preparations of oleic acid emulsions was observed.)

Table 4

Mean Changes Noted in Coagulation Tests Following Infusion of Fatty Acid Solution into Dogs (027)

	Oleic C-18:1	Linolenic C-18:3	Palmitic C-16:0	Stearic C-18:0	Oleic-Palmitic	Oleic-Stearic	Saline
Min. Coag. Time Glass	0.187 *16	0.9 20	-0.26 23	-0.08 12	-1.6 21	-8.0 7	-0.17 18
Min. Coag. Time Silicone	0.91 11	1.8 18	-3.6 18	-1.8 11	-3.2 24	-12.8 5	-1.2 17
Sec. Recal. Time	-1.25 16	-0.8 19	-18.0 17	-15.0 14	-19.2 25	-30.8 6	+2.5 16
Sec. Serum Pro. Time	-1.3 16	-10.5 19	-20.2 21	-25.2 14	-20.3 23	-30.5 9	+0.3 16
Sec. Plasma Pro. Time	-0.31 15	0.05 20	-1.6 19	+0.32 15	-1.6 19	+0.23 9	+0.08 17
Sec. Part. Thromb. Time	+0.02 10	+0.58 18	-3.5 12	+5.0 8	+1.66 9	+2.25 6	-0.40 16
Sec. Hicks-Pitney TGT	+0.06 15	+1.5 4	+0.26 19	+0.83 12	-0.36 11	+0.66 3	-0.4 5

Table 4 (cont.)

	Oleic C-18:1	Linolenic C-18:3	Palmitic C-16:0	Stearic C-18:0	Oleic-Palmitic	Oleic-Stearic	Saline
Stypren Time (1:1) Sec.	+0.22 15	+0.5 19	-3.6 13	-3.5 14	-3.7 26	-1.4 9	+0.43 17
Stypren Time (1:4) Sec.	-0.020 15	+1.3 20	-3.8 16	-3.8 14	-0.4 23	-0.85 9	1.73
Platelet Count	-223,400 8	-174,000 20	-197,000 11	-56,750 8	-177,707 14	-197,000 6	-84.125 16

*Figure in this position indicates number of paired observations made for the fatty acid concerned.

(Note: In interpreting the results, it must be remembered that the infusions were 10 ml/kg into dogs whose blood volume was 92 ml/kg, and a slight dilutional effect occurred due to an increase in blood volume, not saline control.)

Table 5

Mean Changes Produced in Lipid Levels by the Intravenous Infusion of Fatty Acid Solutions into Dogs (027)

	Oleic C-18:1	Linolenic C-16:3	Palmitic C-16:0	Stearic C-18:0	Oleic-Palmitic	Oleic-Stearic	Saline
Total Chol. mg./100 ml.	- 8.0 *14	- 14.6 9	-10.0 17	- 9.0 13	- 7.4 16	- 6.0 11	- 15.4 15
Free Chol. mg./100 ml.	- 6.3 14	- 7.1 10	- 2.0 17	- 8.2 13	- 1.8 16	- 4.0 11	- 3.4 15
Ester. Chol. mg./100 ml.	- 0.5 14	- 7.4 9	- 7.5 17	- 4.4 13	- 4.6 16	- 3.4 11	- 12.0 15
Esters Chol. mg./100 ml.	- 3.0 14	- 12.6 9	- 6.0 16	- 5.1 13	-10.4 16	- 4.7 11	- 19.0 15
Phosphs-Lipids mg./100 ml.	-16.2 14	- 28.6 9	-27.7 18	- 30.0 13	-27.4 15	-13.6 11	- 36.0 15
Tri-Glycer. mg./100 ml.	- 7.0 14	- 6.3 9	+ 0.125 18	- 1.7 13	- 6.1 15	-17.0 11	- 6.4 15
Total Lipids mg./100 ml.	-26.5 14	- 55.2 9	-47.4 18	- 36.4 13	-34.4 16	-31.0 11	- 50.5 15
Non-ester.F.A. µEq./L.	-17.0 16	-219.4 10	-19.4 20	-146.3 9	- 5.9 16	-66.5 11	-187.4 14

*Figure in this position indicates number of paired observations made for the fatty acid concerned.

Single intraperitoneal injections (see Tables 6 and 7 for dosages) were administered to mice (young adults, 17-20 g) ten days before implantation of Bashford adenocarcinoma no. 63 in the left groin. The results (see Tables 6 and 7) indicated that both sodium oleate and oleic acid had a positive effect, producing a material increase in the resistance of mice to subsequent cancer transplant. Results obtained for unsaturated and saturated fatty acids and their salts were compared. The author concluded that unsaturated fats increased the resistance of mice to transplanted cancer, an effect not shared by the saturated fatty acids and their sodium salts. No comparison between results for sodium oleate and oleic acid was made, although the author inferred that it was the oleyl moiety which was responsible for the observed activity.

Nakahara further examined the growth of cancer grafts (also Bashford adenocarcinoma) implanted 2 or 48 hours before an injection of 0.5 ml of 1% solution of sodium oleate or emulsion of oleic acid. Results (see Tables 8 and 9) showed no significant effect on the growth of cancer implanted before injection of the oleic acid or its sodium salt.

Nakahara concluded that sodium oleate was only effective against subsequent cancer grafts and not against grafts already in situ because of the rapidity with which transplantable cancers grew and because artificially induced resistance could only develop after a latent period of several days.

The author suggested the possibility that the resistance to implanted cancer demonstrated by sodium oleate and oleic acid might be consequent on increased activity in the lymphoid tissue. This is evidenced by the observation that injections of sodium oleate, in amounts sufficient

Table 6

Effect of Sodium Oleate on Resistance to Implanted Cancer (090)

Experiment No.	Amount of Sodium Oleate	Treated Mice		Controls	
		No.	Resistant Mice	No.	Resistant Mice
	mg[a]				
1	2	7	2 (28.5%)	9	1 (11.1%)
2	4	12	5 (41.6%)	9	1 (11.1%)
3	6	8	5 (62.5%)	9	2 (22.2%)
4	6	12	4 (33.3%)	10	0 (0.0%)
5	6	16	9 (56.2%)	10	1 (10.0%)
Average for 6 mg.....		36	18 (50.0%)	29	3 (10.3%)

mg[a] = 0.1 ml of 1% solution.

Table 7

Effect of Oleic Acid on Resistance to Implanted Cancer (090)

Experiment No.	Amount of Oleic Acid	Treated Mice		Controls	
		No.	Resistant Mice	No.	Resistant Mice
	cc.				
18	0.004	9	3 (33.3%)	6	1 (16.6%)
19	0.004	10	8 (80.0%)	10	2 (20.0%)
20	0.005	10	7 (70.0%)	12	2 (16.6%)
21	0.006	8	6 (75.0%)	6	1 (16.6%)
22	0.006	11	5 (45.4%)	10	0 (0.0%)
23	0.008	13	8 (61.5%)	11	4 (36.3%)
Average.....		61	37 (60.6%)	55	10 (18.1%)

Table 8

Effect of Sodium Oleate on Cancer Grafts Previously Implanted (090)

Experiment No.	Interval Between Tumor Inoculation and the Soap Injection	Treated Mice		Controls	
		No.	Resistant Mice	No.	Resistant Mice
	hrs.				
38	2	15	7 (46.6%)	10	3 (30.0%)
39	2	7	3 (42.8%)	10	2 (20.0%)
40	48	20	3 (15.0%)	10	0 (0.0%)
41	48	8	1 (12.5%)	8	0 (0.0%)
Average.....		50	14 (28.0%)	38	5 (13.1%)

Table 9

Effect of Oleic Acid on Cancer Grafts Previously Implanted (090)

Experiment No.	Interval Between Tumor Inoculation and Oleic Acid Injection	Treated Mice		Controls	
		No.	Resistant Mice	No.	Resistant Mice
	hrs.				
42	2	15	6 (40.0%)	10	3 (30.0%)
43	48	7	2 (28.5%)	7	1 (14.2%)
44	48	9	4 (44.4%)	10	2 (20.0%)
Average.....		31	12 (38.7%)	27	6 (22.2%)

to produce resistance to implanted cancer brought about an increase in the number of karyokinetic figures in the lymphoid tissue which indicated an increased proliferative activity of this tissue. (For similar experiment, see Palmitic acid, sodium salt, Biological Data, Section IV.)

B. Rabbits

Novikova (095) studied the effect of the simultaneous administration of sodium oleate and cholesterol on the development and course of experimental atherosclerosis in rabbits. Both experimental and control rabbits (male, 2.5-3 kg weight) received 1 g of cholesterol as a 20% solution in sunflower oil every other day in their feed. After one month, 12 experimental animals received 20 ml of a 2% sodium oleate solution three times a week (1.2 g each time) for three months. Ten control animals continued to receive only the cholesterol solution.

The levels of total serum cholesterol and serum phospholipids were investigated every month. The animals were sacrificed four months from the first administration of cholesterol. Total lipids and cholesterol were determined in the aorta and cholesterol levels measured in the liver. The results (see Figures 1 and 2) showed that sodium oleate promoted a fairly rapid increase in alimentary hypocholesterolemia and intensified the deposition of lipids and cholesterol in the aorta and liver. Examination of the aortas showed that they weighed somewhat more on the average in experimental animals (626 $\pm$ 54 mg) than in the controls (587 $\pm$ 52 mg). Gross inspection of the atherosclerotic lesions in the aortas revealed the greatest changes in those animals receiving sodium oleate.

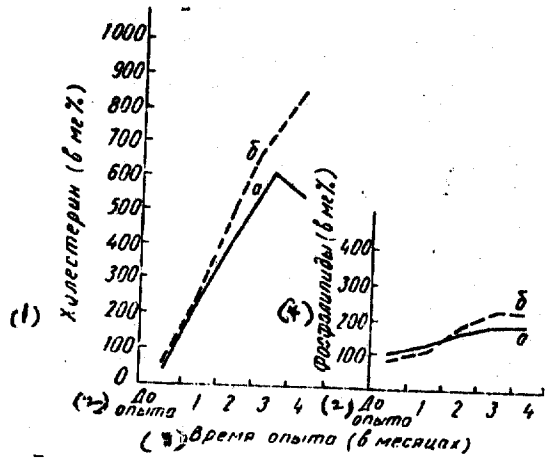


Fig. 1. Effect of sodium oleate on rabbit serum cholesterol and phospholipids in experimental atherosclerosis. (095)

a = control group
b = experimental group
1 = cholesterol (in mg%)
2 = before the experiment
3 = time of the experiment (in months)
4 = phospholipids (in mg%)

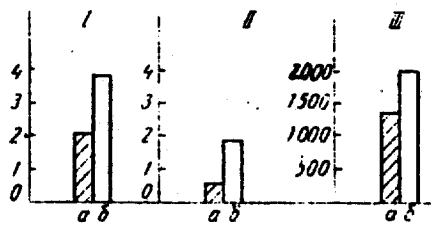


Fig. 2. Average content (in mg%) of total lipids (I) and cholesterol (II) in the aortic wall and cholesterol level of liver tissue (III). (095)

a = control group
b = experimental group

The author suggested that the intensification of experimental atherosclerosis could be caused by the accelerated absorption of exogenous cholesterol and decreased excretion of endogenous cholesterol due to oleic acid; also by increased deposition of cholesterol esters with oleic acid in plaques as other investigators have observed.

C. Cats

Oro and Wretlind (097) injected anaesthetized cats (4 kg weight) intravenously with 1.0% oleic acid emulsion (for composition, see Table 10) and recorded respiratory and pulmonary arterial pressure.

At 50 mg/kg BW oleic acid dosage, a hypotensive effect was observed in the systemic circulation, but a hypertensive effect was observed in the pulmonary artery.

At dose levels less than 50 mg/kg BW, oleic acid had a depressive action on blood pressure sometimes accompanied by hyperpnoea, while doses greater than 50 mg/kg BW resulted in apnoea, reduced blood pressure and convulsions often with a fatal outcome. (Effects were found to be somewhat dependent upon the emulsifier used, e.g., the effect on the pulmonary artery was less pronounced with hydrogenated than with unsaturated phosphatides.)

Vagotomy had no effect on falls in blood pressure and no tachyphylaxis was observed with oleic acid. In several experiments an increased effect was noted upon repeated injection of equal doses. (See Figure 3).

Table 10

Composition of 1% Emulsion of Oleic Acid (097)

Oleic acid	1 g
Cottonseed oil	10 g
Phosphatides	1.2 g
Pluronic F 68 (polypropylene-polyethylene-glucose)	0.4 g
5% glucose solution to	100 ml

(Emulsion adjusted to pH 7.3 with N NaOH)

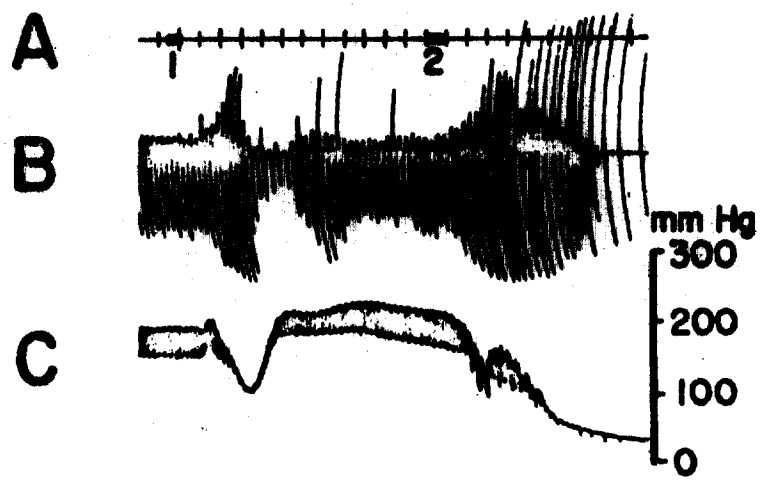


Fig 3. Effect of oleic acid emulsion on blood pressure and respiration. Cat, 4.0 kg, under chloralose anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. One ml/kg of 10% oleic acid emulsion in cottonseed oil (10%) with soyabean phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at 1 and 2. (097)

Biochemical Aspects

I. Breakdown

No information regarding cases of spontaneous breakdown of sodium oleate in food or under conditions of storage, processing or cooking has been in the current literature.

II. Absorption and Excretion

Introduction

Ingested neutral fats (triglycerides) are normally hydrolyzed and absorbed by the intestine as monoglyceride and free fatty acid. Following absorption, triglycerides are reformed in the intestinal mucosa and incorporated into chylomicrons or lipoprotein (very low density) for transportation throughout the organism (026).

In cases where there is an excess of free fatty acid, i.e., where the ratio of absorbed fatty acid to monoglyceride is greater than 2:1, as would be the case in ingesting the free fatty acid, the fatty acid may (after activation with coenzyme A) be combined with alpha-glycerophosphate to form a triglyceride and be incorporated into chylomicrons as above (026).

Rats

In order to study the transportation of oleic acid absorbed by the small intestine, Bergstrom et al. (008) administered 0.5 ml of oleic acid-1 ^{14}C or triolein- ^{14}C via gastric intubation under light anaesthesia to male rats (250 g weight) with cannulated thoracic ducts. To compare the activity of expired CO_2 with that of the cannulated rats, non-cannulated rats were administered labeled oleic acid in amounts corresponding to 0.1 ml/cm^2 body surface via gastric intubation.

After 24 hours, 66-92% (mean 78.2%) of the labeled oleic acid and 57-92% (mean 76.2%) of the labeled triolein were absorbed. Although 38-96% of the absorbed oleic acid was recovered in the thoracic duct lymph, only 2% was in the form of phospholipids. (See Table 11.)

Non-cannulated rats fed labeled oleic acid or labeled methyl oleate expired about 16% of the absorbed activity as CO_2 within 24 hours. Only about 4% expiration was observed in the cannulated group irrespective of administering the oleic acid as the free acid or the triglyceride. (See Figure 4.) The difference corresponds roughly to the loss of labeled material via the fistula and shows that the absorbed labeled compounds are metabolized at a normal rate.

III. Metabolism and Excretion

Introduction

The major pathway through which oleic acid is catabolized is beta-oxidation, consisting primarily of five repeated steps and one special step involving the cis unsaturated portion of oleic acid. All reactions occur in the cell mitochondria. (081, 098)

(a) Activation-- A Long Chain Fatty Acid Thiokinase [Fatty Acid: Co A ligase(AMP)] in the presence of ATP transforms the free oleic acid into an oleyl coenzyme A derivative.

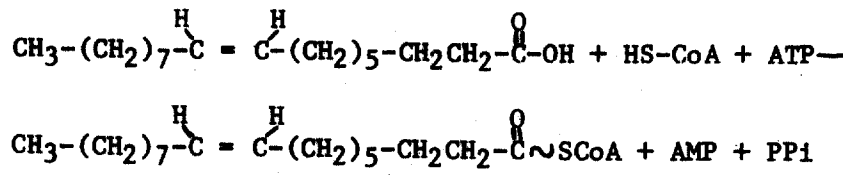


Table 11

Recovery of [^{14}C] Oleic Acid and [^{14}C] Triolein in Thoracic Lymph After Oral Administration to Rats (008)

Rat No.	Substance fed	Amount of administered activity absorbed (%)	Amount of absorbed activity recovered in lymph as fatty acids (%)	Amount of lymph fat activity found in: Glyceride fatty acids (%)	Amount of lymph fat activity found in: Phospholipid fatty acids (%)	Amount of absorbed activity in expired CO_2 (%)
1	Oleic acid	91.5	64.0	98.2	1.8	4.8
2	Oleic acid	65.5	38.1	98.8	1.2	3.7
3	Oleic acid	73.0	96.4	97.4	2.6	3.5
4	Oleic acid	91.8	62.5	97.9	2.1	---
5	Oleic acid	69.0	53.8	97.5	2.5	4.5
Mean		78.2	63.0	----	---	---
6	Triolein	92.1	59.3	97.5	2.5	4.4
7	Triolein	56.5	50.5	97.7	2.3	5.0
8	Triolein	85.0	69.2	97.4	2.6	4.0
9	Triolein	71.0	82.9	98.8	1.2	---
Mean		76.2	65.5	----	---	---

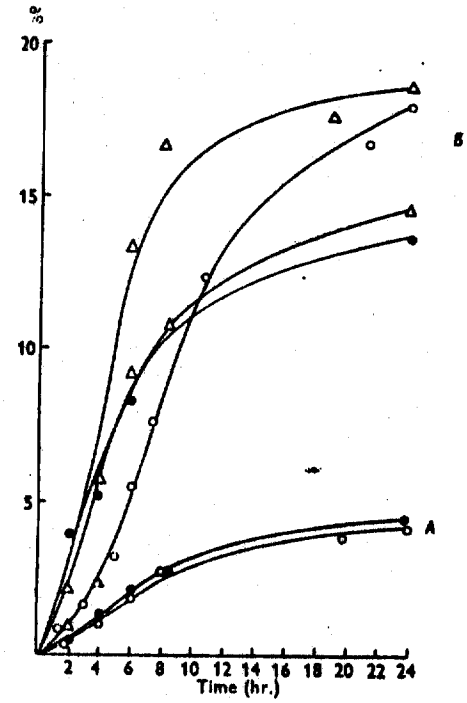
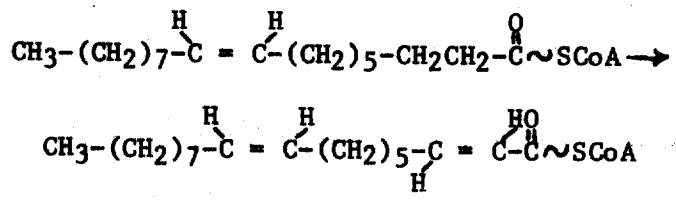


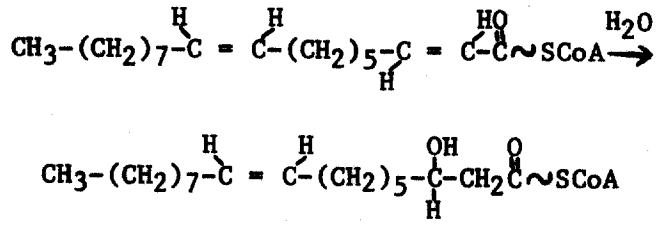
Fig. 4 Percentage of absorbed isotope in expired CO_2 after feeding [^{14}C] oleic acid as triolein (O), methyl oleate (Δ) or free oleic acid (O). A, rats with lymph fistula; every point represents the mean of three animals. B, rats without fistula; each point represents a single animal. (008)

(b) Dehydrogenation-- The appropriate fatty acyl CoA dehydrogenase desaturates the oleyl coenzyme A derivative in the alpha and beta positions, resulting in a <u>trans</u> configuration at this double bond.

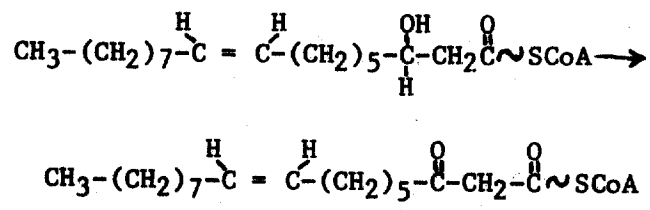


The coenzyme FAD is reduced in this reaction.

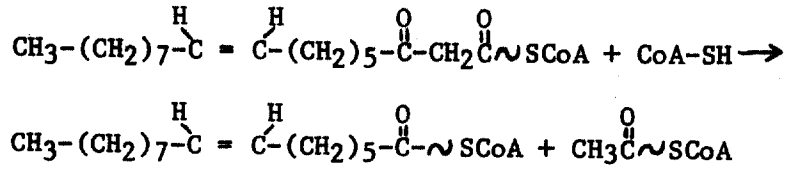
(c) Hydration-- One molecule of water is added across the newly formed double bond in the presence of enolhydratase (3-hydroxy Co A hydrolase), forming the beta-hydroxyloleyl Co A derivative of L configuration.



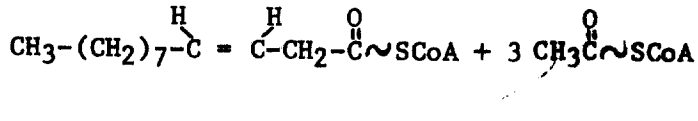
(d) Oxidation (2nd dehydrogenation)-- L-3-hydroxy fatty acyl Co A dehydrogenase oxidizes the hydroxyl group of the beta-hydroxyoleyl Co A derivative to a keto group. The coenzyme NAD^+ is reduced to NADH.



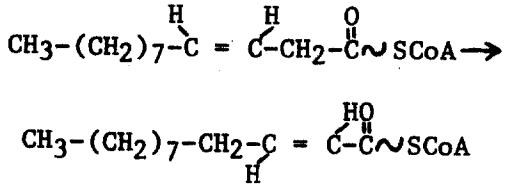
(e) Thiolytic cleavage-- Beta-ketothiolase breaks the beta-keto derivative to form acetyl Co A and then adds one molecule of coenzyme A back to the fatty acid forming an activated fatty acid which is 2 carbons shorter than the original oleic acid.



The shortened fatty acyl Co A derivative repeats the cycle (b), (c), (d), (e) twice more resulting in the products:



This fatty acyl Co A derivative is <u>cis</u>-beta, gamma unsaturated and must be converted to the <u>trans</u>-alpha, beta unsaturated compound, a transformation which occurs in the presence of the enzyme $\Delta^{3,4}$<u>cis</u>-$\Delta^{2,3}$<u>trans</u>-enoyl: Co A isomerase.



The product is analogous to that in (b) and the cycle is then repeated until the oleic acid is completely converted into acetyl Co A (9 molecules)

which may condense with oxaloacetic acid and be oxidized through the Krebs tricarboxylic acid cycle and eventually leave the body as exhaled CO_2.

No information directly concerning oleic acid or sodium oleate metabolism and excretion was noted in the current literature.

IV. Effects on Enzymes and Other Biochemical Parameters

In vitro

1. Because the sodium salts of long chain fatty acids were reported to accelerate blood clotting and to cause massive thrombosis in dogs, Surgala et al. (121) undertook a study of the fibrinolytic activity of the salts of fatty acids with special regard to the influence of saturation and unsaturation.

To Petri dishes prepared with 10 ml bovine fibrinogen (2.5 mg/ml) and 0.5 ml bovine thrombin (50 NIH units/ml) in sodium borate buffer, pH 7.7, was added 0.02 ml of 2-fold dilutions of the salts of fatty acids. The lowest concentration causing liquefaction sufficient for complete perforation of the fibrin film after incubation at 37° C for at least 4 hours was taken as the endpoint.

Table 12 presents the results. The authors suggested that the unsaturated fatty acid salts, of which the oleate is representative, were more fibrinolytic than the corresponding saturated fatty acid salts. The authors noted a possible role of fatty acids in regulation of fibrin formation and digestion. (For similar experiment, see Palmitic acid, sodium salt, Biochemical Aspects, Section IV, In vitro, 1.)

2. Kerr et al. (065) tested the effect of fatty acids on platelet aggregation. 10 mg (approx.) of the sodium salt of oleic acid were powdered and slowly added to 10 ml of purified lecithin solution, giving

Table 12

Influence of Unsaturation on Fibrinolytic Activity (121)

Fatty Acid		Active concentration, mM — Test No.[a] 1	2	3	4	5
Palmitate	C16:0		5	.3	.3	
Palmitoleate	C16:1		.5	.3	.3	
Stearate	C18:0		.6	1	1	
Oleate	C18:1	.2	.06	.2	.08	
Elaidate	C18:1		.06	.2	.2	
Arachidate	C20:0		1	5	1	
Eicosenoate	C20:1					.08
Behenate	C22:0		>10	>10	>10	
Erucate	C22:1		.03	.02	.04	
Lignocerate	C24:0					>10
Nervonate	C24:1					.08

a = Test 1: sodium salt
Test 2-5: potassium salt

a 0.0035 M oleate-lecithin sol. 0.5 ml of the sol was added to 1.5 ml of platelet-rich plasma (blood drawn from humans) and the optical density measured for platelet aggregation. The oleate-lecithin sol caused immediate (but transient) platelet aggregation comparable with palmitate-lecithin and myristate-lecithin sols. Figures 4a and 4b show the results of the oleate compound compared to other unsaturated and saturated fatty acid sols. (For a similar experiment, see Palmitic acid, sodium salt, Biochemical Section IV, In vitro, 2.)

3. In a one-stage clotting mixture with human citrated, platelet-poor plasma and Russell's viper venom, Korsan-Bengsten et al. (068) showed that sodium oleate promoted clotting.

4. Yamamoto (142) reacted sodium oleate with isolated rat mitochondria both with and without bovine serum albumin (BSA). He determined the effect on mitochondrial function and structure by measuring: oxygen uptake; 90° light scattering (a means of measuring structural changes in the mitochondria); ATPase activity; and fluorescence of pyridine nucleotides. The following results were obtained.

(a) The oleate at a concentration of 0.1 μmole/mg protein caused the greatest shrinkage of 1 mg mitochondrial protein and oxidation of intramitochondrial pyridine nucleotides. At an oleate concentration of more than 0.1 μmole per mg mitochondrial protein, the degree of shrinkage was reduced in proportion to the increment of oleate concentration. At an oleate concentration of more than 0.2 μmole per mg protein, prompt swelling of mitochondria without shrinkage was induced, accompanied by the inhibition of respiration.

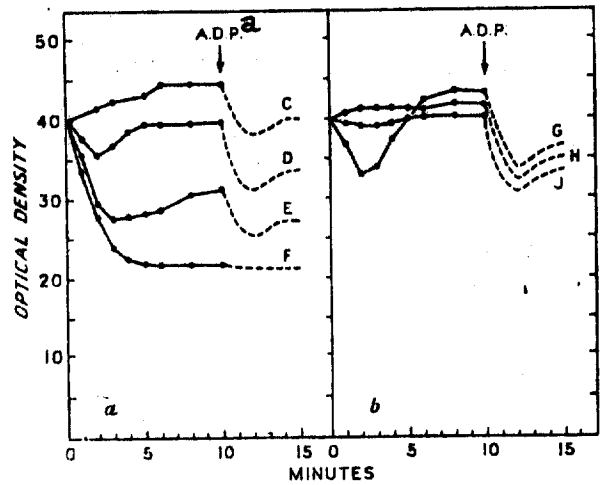


Fig. 4a - Effect of various sols of lecithin with saturated fatty acids on platelet-rich plasma. Means of 8 patients.
C - No fatty acid.
D - Myristate (14-carbon).
E - Palmitate (16-carbon).
F - Stearate (18-carbon).

Fig. 4b - Effect of various sols of lecithin with unsaturated fatty acids on platelet-rich plasma. Means of 8 patients.
G - Oleate (18:1-carbon).
H - Linolenate (18:3-carbon).
J - Linoleate (18:2-carbon).

A.D.P.[a] (adenosine triphosphate) 1 or 2 mg in 0.01 ml barbiturate buffered saline added to assess viability of the platelets. (065)

(b) The shrinkage and uncoupling of mitochondria and the oxidation of the nucleotides could be readily reversed by BSA.

Yamamoto felt that as yet there was no satisfactory explanation for the mechanism of the action of oleate on mitochondria.

5. Cameron (018) mixed fresh human saliva with sodium oleate (or oleic acid) and starch, and observed the action of the oleate on the enzyme diastase by the Mohr-Bertrand method.

The results obtained showed that sodium oleate decreased the enzyme's activity, an effect which was directly proportional to the length of time of action and concentration of the oleate.

6. Reyner (106) found that both the surface tension of whole blood and the rate of sedimentation of red blood cells were decreased by the addition of sodium oleate.

7. Because evidence of in vitro hemolysis has been reported following alimentary hyperlipemia and other conditions associated with rapid increases in serum fatty acids, Greisman (043) studied the interaction of sodium oleate with human erythrocytes.

Using phosphate buffered solutions of erythrocytes from normal humans and sodium oleate, the author found that the oleate induced lysis and that:

(a) At concentrations less than 8×10^{-4} M (pH 7.2 ± 0.1), the initial step in human erythrocyte lysis involves erythrocyte-oleate binding. Oleate binding by a standard number of erythrocytes in a fixed volume is a function of oleate concentration, temperature and contact time.

(b) The saturation level appears to be 111×10^{-9} μg sodium oleate/erythrocyte (36.5×10^{-17} moles/erythrocyte); a mean minimum of

approximately 7 x 10^{-9} μg sodium oleate/erythrocyte (2.3 x 10^{-11} moles/erythrocyte) is required (at 37°C, pH 7.1) for lysis rates detectably faster than controls. Once hemolytic quantities of sodium oleate are bound, hemolysis (at pH 7.2 ± 0.1) proceeds as a function of quantity, bound and temperature. (See Figures 5, 6, and 7)

(c) Observed lowered surface tension of erythrocytes resulting from contact with oleate solution does not influence hemolysis rates under experimental conditions.

(d) Non-specific enzyme inhibitors (fluoroacetate, fluoride and azide) at final concentrations of 0.01 M did not alter the hemolytic activity of sodium oleate. Cyanide ion did retard oleate hemolysis, though it was not known if this was related to enzymatic interference.

(e) Hemolytic substances are released following oleate hemolysis which may be oleate or oleate-erythrocyte interaction products.

On the basis of (e), the author suggested that oleate possesses the potential of inducing a decelerating hemolytic chain reaction. (For a similar experiment, see Palmitic acid, sodium salt, Biochemical Aspects, *In vitro*, 3.)

In vivo

A. Rats

1. Rochman *et al.* (108) administered sodium oleate (for dosages, see Table 13) intravenously or intraperitoneally to rats with a pyloric ligation. Injections followed 48 hour fasting. Animals were sacrificed four hours after injection. Gastric secretions were measured according to the method of Shay (Gastroenterology 26:906, 1954). The results (see Table 13) showed

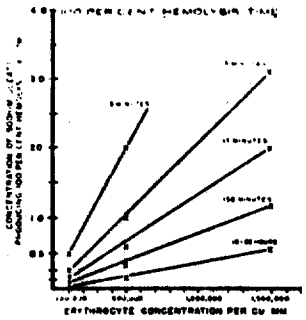


Fig. 5

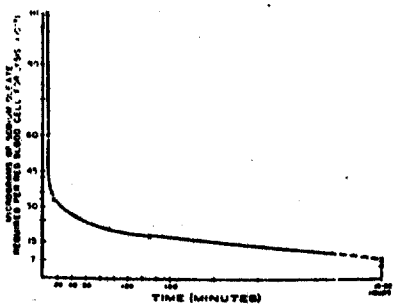


Fig. 6

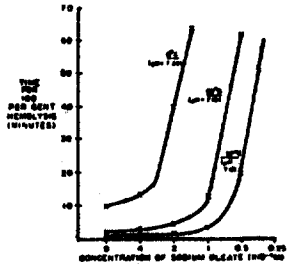


Fig. 7

Fig. 5 - Relation of human erythrocyte concentration to concentration of oleate required to induce 100% hemolysis at comparable rates at 37°C, pH 7.1. (043)

Fig. 6 - Relation of quantity of sodium oleate available per human erythrocyte (in 1.0 ml vol) to 100% hemolysis time at 37°C, pH 7.1. Data derived from Fig. 5. (043)

Fig. 7 - Effect of temperature on 100% hemolysis time of human erythrocytes exposed to varying concentrations of oleate. Each value represents mean of 5 determinations. (043)

Table 13

Administrations to Rats (108)

Substance	No. of rats	Method of administration	Avg. ml secretion/100 g
Normal saline	24	i.p.	3.6
Distilled water	31	i.p.	3.1
Nothing	26	i.p.	3.7
0.1 N sodium oleate[a]	36	i.p.	2.0
0.1 N sodium oleate[a]	29	i.p.	1.9
0.2 N sodium oleate[b]	24	i.p.	1.7
Normal saline	26	i.v.	3.0
0.1 N sodium oleate[a]	25	i.v.	1.9
0.2 N sodium oleate[b]	25	i.v.	1.5

[a] = 7.5 mg sodium oleate
[b] = 15.0 mg sodium oleate

that sodium oleate decreased the volume of gastric secretion by one-third over a four hour period.

The authors suggested that the secretory inhibition was caused by sodium oleate after its absorption into the blood stream and was independent of contact by sodium oleate with the intestinal mucosa.

2. Kowalewski (069) studied the inhibitory effect of sodium oleate on gastric secretory function in nonstimulated and histamine stimulated rats.

Solutions (for composition, see Table 14) were infused intravenously into cannulated rats at a rate of 120 ml/kg BW for 24 hours. Gastric juice was collected simultaneously. The effects of the infusions are shown in Tables 14 and 15.

It was found that sodium oleate suppressed the volume of gastric secretion and the output of acid and pepsin in nonstimulated and histamine stimulated rats. The author noted that contact of sodium oleate with the duodenal mucosa is not a necessary condition for the suppression of gastric secretory function, lending support to the work of Rochman et al. above.

B. Dogs

Crespin et al. (023) used mongrel dogs of both sexes after an overnight fast and infused sodium oleate into their plasma. The following results were obtained (see also Table 16).

(a) Acute elevation of plasma insulin within 10 minutes after the start of infusion.

(b) Marked fall in plasma glucose even after some dogs received intravenous glucose.

(c) Significant increase in insulin levels in pancreaticoduodenal vein blood.

(d) Slight increase in plasma ketone levels.

Table 14

Effect of Intravenous Infusion of Sodium Oleate and/or Histamine on 24 h Output of Various Components of Gastric Juice in Rats. Av. values and S.D. (069)

Group	Infusion	No. of rats	B.W. g	Vol. ml	HCl mEq	Na^+ mEq	K^+ mEq	Cl^- mEq	Pepsin mg
1	Normal saline	10	232 ± 6	14.1 ± 3.8	0.97 ± 0.4	0.70 ± 0.19	0.25 ± 0.09	0.91 ± 0.18	362 ± 174
2	.2N sodium oleate in normal saline (1:3)	10	231 ± 5	7.0 ± 2.0	0.38 ± 0.2	0.51 ± 0.20	0.12 ± 0.05	0.81 ± 0.33	232 ± 120
3	Histamine cl. hydrochloride in normal saline (.83 mg/ml)	10	232 ± 16	23.9 ± 7.3	2.69 ± 1.0	0.76 ± 0.28	0.25 ± 0.08	1.59 ± 0.68	578 ± 230
4	Histamine and sodium oleate in saline	15	230 ± 16	7.4 ± 6.5	0.72 ± 0.7	0.40 ± 0.38	0.22 ± 0.30	1.32 ± 0.95	186 ± 178

Table 15

Effect of Intravenous Infusion of Sodium Oleate and/or Histamine on the Concentration of Various Components of Gastric Juice, Collected or 24 h in Rats. Same Rats as in Table 14. Av. Values and S.D. (069)

Group	Infusion	pH	mEq/l				Pepsin mg/ml
			HCl	Na^+	K^+	Cl^-	
1	Saline	1.68 ± 0.57	66 ± 20	51.5 ± 13.0	17.3 ± 3.8	68.0 ± 17.5	25.5 ± 10.6
2	Sodium oleate in saline	3.18 ± 2.10	52 ± 31	75.9 ± 25.4	17.0 ± 5.7	114.0 ± 20.2	32.7 ± 12.0
3	Histamine in saline	1.21 ± 0.06	112 ± 20	33.8 ± 15.5	10.8 ± 2.6	66.6 ± 22.7	23.8 ± 3.8
4	Histamine and sodium oleate in saline	1.52 ± 0.50	96 ± 35	58.1 ± 29.7	14.3 ± 8.6	164.0 ± 8.6	21.4 ± 8.0

Table 16

Summary of Effects of Oleate Infusion on Plasma FFA, Glucose, and Insulin* (023)

Exp. No.	Dog	Infusion rate	Mean plasma FFA: Control	Mean plasma FFA: Infusion	Mean plasma FFA: Δ	Mean plasma glucose: Control	Mean plasma glucose: Infusion	Mean plasma glucose: Δ	P value	Mean plasma insulin: Control	Mean plasma insulin: Infusion	Mean plasma insulin: Δ	P value
		μEq/kg per min	μEq/ml			mg/100 ml				μU/ml			
High elevation of plasma FFA													
1	A	58.3	0.59 ±0.02	1.80 ±0.12	1.21 ±0.12	109 ±4	71 ±8	38 ±9	<0.01	7 ±1	31 ±5	24 ±5	<0.01
2	B	59.0	0.87 ±0.05	1.94 ±0.08	1.07 ±0.11	106 ±2	52 ±2	54 ±3	<0.01	6 ±1	72 ±55	66 ±56	NS
3	C	64.8	0.66 ±0.05	1.87 ±0.08	1.21 ±0.09	111 ±6	66 ±5	45 ±8	<0.01	8 ±3	64 ±3	56 ±4	<0.01
4	C	43.3	0.65 ±0.08	1.62 ±0.10	0.97 ±0.12	121 ±4	83 ±3	38 ±5	<0.01	26 ±5	115 ±25	89 ±25	<0.05
5	D	40.8‡	0.50 ±0.03	1.41 ±0.02	0.91 ±0.04	92 ±2	71 ±2	21 ±3	<0.01	6 ±1	49 ±13	43 ±13	<0.05
6	E	35.5§	0.36 ±0.03	1.57 ±0.03	1.21 ±0.04	123 ±2	95 ±1	28 ±3	<0.01	12 ±1	28 ±3	16 ±3	<0.01
7	D	29.5§	0.31 ±0.02	1.40 ±0.04	1.09 ±0.04	143 ±3	87 ±6	56 ±6	<0.01	14 ±3	43 ±5	29 ±6	<0.01
Mean ±SE		47.3 ±5.1	0.56 ±0.07	1.66 ±0.08	1.10 ±0.11	115 ±6	75 ±5	40 ±8		11 ±3	57 ±11	46 ±12	
Low elevation of plasma FFA													
8	F	37.2	0.56 ±0.03	1.05 ±0.07	0.49 ±0.07	106 ±3	90 ±6	16 ±7	NS	4 ±1	12 ±1	8 ±2	<0.01
9	D	36.0	0.41 ±0.01	0.99 ±0.05	0.58 ±0.05	131 ±3	99 ±0	32 ±3	<0.01	7 ±1	16 ±1	9 ±1	<0.01
10	F	24.8	0.57 ±0.04	0.98 ±0.03	0.41 ±0.05	106 ±1	98 ±1	8 ±1	<0.01	7 ±1	16 ±1	9 ±1	<0.01
11	G	19.4	0.55 ±0.05	1.07 ±0.05	0.52 ±0.07	114 ±2	103 ±2	11 ±3	<0.05	8 ±1	15 ±1	7 ±2	<0.01
Mean ±SE		29.4 ±1.3	0.52 ±0.04	1.02 ±0.02	0.50 ±0.04	114 ±6	97 ±3	17 ±6		7 ±1	15 ±1	8 ±1	
Simultaneous glucose infusion, *100 mg/min*													
12	H	32.1	0.34 ±0.01	1.33 ±0.04	0.99 ±0.04	128 ±3	103 ±1	25 ±4	<0.01	16 ±1	37 ±3	21 ±3	<0.01
13	I	45.7	0.34 ±0.04	2.02 ±0.07	1.68 ±0.09	139 ±2	65 ±2	74 ±3	<0.01	13 ±1	83 ±11	70 ±12	<0.01
14	J	34.8	0.37 ±0.03	1.42 ±0.03	1.05 ±0.05	153 ±3	99 ±4	54 ±5	<0.01	31 ±8	121 ±44	90 ±44	NS
15	K	43.6‡	0.45 ±0.04	1.67 ±0.04	1.22 ±0.05	135 ±5	93 ±1	42 ±5	<0.01	13 ±1	31 ±4	18 ±4	<0.01
16	L	80.0‡,‖	0.42 ±0.01	1.85 ±0.11	1.43 ±0.11	151 ±2	134 ±1	17 ±3	<0.01	39 ±2	725 ±99	686 ±99	<0.01
17	M	31.7¶	0.42 ±0.05	1.07 ±0.05	0.65 ±0.07	359 ±9	318 ±9	41 ±13	<0.05	76 ±1	156 ±22	80 ±22	<0.05
Mean ±SE		39.1 ±3.3	0.38 ±0.03	1.61 ±0.16	1.23 ±0.05	139 ±5	90 ±9	49 ±10		18 ±4	68 ±21	50 ±22	
Sodium Bicarbonate infusion, *140 μEq/kg per min*													
18	N	0	0.38 ±0.02	0.32 ±0.02	0.06 ±0.03	150 ±2	117 ±1	33 ±2	<0.01	40 ±4	21 ±3	−19 ±6	<0.05
Pancreatic vein study													
19	PV-1	19.6§	0.23 ±0.02	1.60 ±0.11	1.37 ±0.11	126 ±6	137 ±1	11 ±6	NS	FA 5 ±1	18 ±0	13 ±1	<0.01
										PV 147 ±32	399 ±47	252 ±57	<0.01
20	PV-2	36.5§	0.37 ±0.02	1.48 ±0.06	1.11 ±0.07	137 ±3	99 ±7	38 ±8	<0.01	FA 5 ±1	173 ±33	168 ±33	<0.01
										PV 55 ±6	3544 ±661	3489 ±661	<0.01

FA, femoral artery; PV, pancreaticoduodenal vein; NS, not significant; FFA free fatty acids.

*Control values are means ± SEM of five separate determinations during the one hour of saline infusion which preceded each oleate infusion. FFA infusion values are means ± SEM of all FFA determinations during infusion, while glucose and insulin values are means ± SEM of three determinations during the 30 minute infusion period of maximum response for glucose and insulin, respectively.

‡Glycerol combined with oleate (1:3 molar ratio).

§Dog anesthesized with pentobarbital (30 mg/kg).

‖Extraordinary rate; values not included in group means.

¶Glucose infusion at 500 mg/min; values not included in group means.

V. Drug Interaction

Rats

Lecoq *et al*. (077) were able to reverse the toxic effects (shortened nervous chronaxia, prolonged muscle chronaxia, increased excitability of the central nervous system) in rats ingesting 20-22% sodium oleate (see Biological Data, II, A, 1) by oral administration of nicotinamide, pantothenic acid or pyridoxine (daily doses of 100 μg).

VI. Consumer Exposure

As discussed earlier, (see Chemical Information, Section VIII) the sodium salts of oleic and palmitic acid are produced by the alkaline hydrolysis of neutral fats (triglycerides). These salts may be used as food additives when applied as binders, emulsifiers, or anti-caking agents provided they are prepared from fatty acids which conform to food grade specifications.

No information regarding production levels, annual poundage or possible daily intake is available. Percentage use in food categories was not given in the NAS/NRC tables, however, the WITCO Chemical Corporation (141) suggested use of sodium oleate as a thickener and emulsifier for aqueous systems at 1-5% levels.

PALMITIC ACID, SODIUM SALTS

CHEMICAL INFORMATION

I. **Nomenclature**

A. Common names: Palmitic acid, sodium salt, Sodium palmitate, Sodium palmate, Sodium palmitinate

B. Chemical names: Hexadecanoic acid, sodium salt

C. Trade names: none

D. Chemical Abstracts Service Unique Registry Number: 408355

II. **Empirical Formula**

$C_{16}H_{31}O_2Na$

III. **Structural Formula**

$CH_3(CH_2)_{14}\overset{O}{\overset{\|}{C}}ONa$

IV. **Molecular Weight**: 278.31

V. **Specifications**

A. The 13th Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (060) presents the following specifications regarding food use of salts of saturated fatty acids, including sodium palmitate:

1. Definition

Scope: The Na, K, NH_4, Ca, Mg, and Al salts of commercial myristic, palmitic, and stearic acids produced from edible fats and oils.

Functional Use in Food: Anti-caking agents

Description: The products occur as hard, white or faintly yellowish, somewhat glossy and crystalline solids, or as white or yellowish-white powder.

2. Characteristics

Identification:

a. Solubility: Sodium, potassium and ammonium salts

Water: soluble

Ethanol: soluble

b. Heat 1 g with a mixture of 25 ml water and 5 ml hydrochloric acid. Fatty acids are liberated, floating as a solid oil layer on the surface which is soluble in hexane. After cooling, the aqueous layer is decanted, evaporated to dryness, and the residue gives positive tests for the appropriate cation.

Sodium: Add uranyl zinc acetate T.S.; a yellowish crystalline precipitate appears within a few minutes.

Specification:

The article of commerce can be specificed by gas or thin layer chromatography of the fatty acids obtained from the salts, saponification value, free fatty acids, solidification point for the fatty acids obtained from the salts, iodine value, residue on ignition including assay of the cation, unsaponifiable matter and moisture content.

Limits of Impurities:

Arsenic: not more than 3 ppm.

Heavy metals (as Pb): not more than 10 ppm.

Examination:

Arsenic: Prepare test solution as directed for organic compounds.

Heavy metals: Prepare and test a 2 g sample as directed under Method II in Heavy Metals Test, using 20 mcg lead ion in the control (Solution A).

B. The WITCO Chemical Corporation lists the following specifications for

sodium palmitate, technical grade (141):

	Min.	Max.
Free Fatty Acid	0.2	1.3
Moisture	1.5	2.5
Ash (as Na_2CO_3)	17.0	17.5
Free Alkalinity	-- None	--
Fineness -- 100 Mesh, %	40	--
Color	Equal to Standard	

VI. Description

A. General Characteristics:

No information was available specifically for sodium palmitate, however, the sodium salts of long chain fatty acids generally occur as white or faintly yellow crystalline solids or as white or yellowish-white powders.

B. Physical properties:

Sodium palmitate is soluble in both water and alcohol.

C. Stability in containers:

Sodium salts of long chain saturated fatty acids are generally stable in closed containers for long periods of time under normal conditions of temperature and humidity.

VII. Analytical Methods

Although the separation and identification of individual fatty acids is generally a difficult matter, common separation techniques, such as thin-layer chromatography, silica gel and gas-liquid chromatography are applicable.

The following methods of analysis are quantitative for salts (generally sodium) of long chain fatty acids (palmitic, oleic, stearic, etc.):

1. The Official Methods of Analysis of the Association of Official Agricultural Chemists (AOAC), 10th Edition (002) has given official status to a method for quantifying soaps (salts of fatty acids).

The sample in petroleum ether, is extracted several times with 50% alcohol. The alcohol layers are combined and evaporated. The residue is dissolved in H_2O made alkaline and extracted with ether. Ether layers are combined and evaporated. The residue is weighed as fatty acids. From the weight of the fatty acids, the percentage of soap in the sample is calculated as sodium or potassium oleate.

Note is made that error will result if the apparent molecular weight of fatty acids differs appreciably from that of oleic acid.

2. The Standard Methods of Chemical Analysis (138) presents a method in which 75-100 g of sample are ashed, and the ash added to 50 ml of cold, boiled distilled water. The solution is titrated with 0.02 N acid to a methyl orange endpoint.

$$\% \text{ Soap as sodium oleate} = \frac{\text{ml of } 0.02\ \underline{N} \text{ acid} \times 0.608}{\text{Wt. of sample}}$$

3. The Standard Methods of Chemical Analysis (138) also presents a titrimetric method for the quantitative analysis of soaps. Hydrochloric acid 0.01 N is added to a 100 ml solution of acetone containing 2% water and 0.5 ml of 0.1% bromophenol blue in 95% alcohol to the first appearance of a permanent yellow color. A test tube containing 40 g of sample and 1 ml of water is warmed and shaken. 50 ml of the neutralized acetone solution is added (again with warming and shaking), and the mixture is then allowed to stand until layers separate. Green or blue in the upper layer indicates the presence of soap.

The upper layer is titrated with 0.01 N hydrochloric acid to the

reappearance of the yellow color.

$$\% \text{ soap as sodium oleate} = \frac{\text{ml of } 0.01\ \underline{N} \text{ acid} \times 0.304}{\text{Wt. of sample}}$$

This method is reported to be satisfactory for determining soap in oil within the range of 10-500 ppm.

VIII. Occurrence

Oleic and palmitic acids do not normally occur as their sodium salts, but rather as triglyceride esters in natural fats. The sodium (or potassium) salts of these acids, or soaps as they are commonly referred to, are the product of saponification of animal and vegetable fats and oils; that is, alkaline hydrolysis of the fatty acid esters usually with sodium (or potassium) hydroxide.

Palmitic acid, which may be obtained from palm oil, Japan wax, or Chinese vegetable tallow is characteristic of saturated fats, occurring usually as 15-50 % of the fat. It is found to be absent from natural fats only in a very few instances.

In the animal organism, the fatty acids are stored and transported as neutral fats (triglycerides) or phospholipids. In the blood there may be a small amount of free fatty acid, which will be present as soaps (098) due to the alkaline pH and the presence of cations such as sodium and potassium.

BIOLOGICAL DATA

I. Acute Toxicity

A. Mice

Oro and Wretlind (097) injected a 0.2% emulsion (for composition, see Table 17) of palmitic acid intravenously into the tail vein of mice, at least 6 groups of 10 animals each (13-14 kg weight). An LD_{50} value of 57 ± 3.4 mg/kg was found for palmitic acid. (See results of a similar experiment, oleic acid, sodium salt, Biological Data, Section I, A).

Table 17
Composition of 2% Emulsion of Palmitic Acid (097)

Palmitic acid	0.2 g
Cottonseed oil	10 g
Phosphatides	0.4 g
Sodium cholate	0.1 g
Glycerol monostearate	0.5 g
5% glucose soution to	100 ml

(Emulsion adjusted to pH 7.3 with N NaOH)

B. Dogs

1. Cucuianu et al. (024) infused sixteen mongrel dogs (12 males, 4 females, weighing 9-20 kg) intravenously with 6.8 mg/kg sodium palmitate emulsion over a 7 minute period. Six dogs died within 6-160 minutes following the injection with sudden asphyxia and circulatory insufficiency.

Blood was drawn from the dogs and examined. There were decreases in platelet counts, platelet adhesiveness, and fibrinolytic activity (see Table 18).

Table 18

Results from Infusing Dogs with Sodium Palmitate Emulsion (024)

No. of dogs	Sex	Platelet count x 10^3 I[a]	Platelet count x 10^3 II[b]	Adhesiveness I[a]	Adhesiveness II[b]	Fibrinolytic Activity I[a]	Fibrinolytic Activity II[b]
1	M	500	160	70	23	60	30
1	M	652	106	41	24	70	30
1	M	336	107	55	20	60	20
1	M	331	80	48	16	30	10

[a]Initial values.
[b]15 minutes after the beginning of the infusion.

2. Day, et al. (027) studied the effects on dogs of a single rapid infusion of sodium palmitate. Intravenous infusions of various fatty acids in doses of 10 mg/kg BW (as a 0.1% suspension of their sodium salts) were administered to 195 male mongrel dogs judged to be healthy and weighing an average of 10 kg. A 33% mortality rate was observed in the dogs receiving sodium palmitate. When palmitate was infused as a mixture with sodium oleate, the mortality rate dropped to 10% (see Table 20).

Massive venous and arterial thrombi were found in 8 of 11 necropsied palmitate infused animals. The authors concluded that when infused intravenously in dogs over a five minute period in doses of 10 mg/kg as a 0.1% suspension of their sodium salts, the saturated fatty acids (palmitate) were highly thrombogenic as compared to the unsaturated fatty acids. (See results of similar experiments, oleic acid, sodium salt, Biological Data, Section I, B).

Table 19

Acute Toxicity

(by substance or constituent thereof)

Substance	Animal (Species)	Sex & No. (M or F)	Route (p.o.,i.v.,s.c., i.p.,i.m., other)	Dosage mg/kg body wt.	Measurement (LD50, ED50 or other	Ref. Bibliogr. No.
Palmitic acid	Mice	at least 6 groups of 10 animals per group	i.v.	57 ± 3.4	LD_{50}	Oro and Wretlind (097)
Sodium palmitate	Dogs	M, 12 F, 4	i.v. (rapid infusion)	6.8	37.5% mortality	Cucuianu, et al. (024)
Sodium palmitate	Dogs	M, 45	i.v. (rapid infusion)	10	33% mortality	Day, et al. (027)

Table 20

Acute Toxicity of Sodium Palmitate and Other Fatty Acids (027)

Substance (sodium salt)	No. of dogs	Sex	Route	Dosage (mg/kg BW)	% Mortality
Palmitate	45	M	i.v.	10	33
Oleate	29	M	i.v.	10	0
Linoleate	20	M	i.v.	10	0
Stearate	36	M	i.v.	10	25
Oleate-palmitate	20	M	i.v.	10	10
Oleate-stearate	28	M	i.v.	10	71
Saline (control)	17	M	i.v.	10	0

The authors concluded that the results indicated that the decrease in platelet counts was a possible cause of death but did not prove that death was produced by thrombosis.

II. Short-Term Toxicity

A. Rats

Herting et al. (055) fed Holtzman weanling rats (groups of 48-72, with equal numbers of males and females in each group) palmitic acid over a 24-week period. Control rats were fed a diet of 4% corn oil, plus 10% triacetin.

After 24 weeks, the rats received instead a diet of 20% corn oil for another 24 weeks. Rats were sacrificed at 8 week intervals and their tissues were examined. The incidence and severity of lipogranuloma is summarized in Table 21.

Table 21

Incidence and Severity of Lipogranuloma in Rats
Fed Palmitic Acid (055)

Fat	Saturated fatty acid (% of diet)	Time (weeks)	Rats	Reaction in perigonadal fat none	slight	moderate	severe
Palmitic acid	50.4	8	6	3	0	1	1
		16	6	2	0	2	2
		24	5	1	1	2	1

It could be seen that lipogranuloma, a foreign-body-type reaction in adipose tissue, was rapidly produced in rats fed palmitic acid. Replacing the palmitic acid diet, however, with 20% corn oil, led to prompt diminution (evident in all groups after 8 weeks), and eventual disappearance of the lipogranuloma (see Table 22). After the reversal of the reaction, the adipose tissue showed no signs of damage.

The authors concluded that lipogranuloma was caused by excessive intake of the saturated long-chain fatty acid, palmitic acid; its effects were remedied by replacing the saturated fat diet with a diet of unsaturated fat in the form of corn oil.

Table 22

Effect of Replacing Palmitic Acid with Corn Oil (055)

Former dietary fat	20% corn oil (wk)	Rats	Reaction in perigonadal fat none	slight	moderate	severe
Palmitic acid	0	5	1	1	2	1
	8	5	3	1	0	1
	16	2	2	0	0	0
	14	1	1	0	0	0

B. Dogs

1. Cucuianu et al. (024) studied the effect of repeated infusions of sodium palmitate on dogs. Sixteen mongrel dogs (12 males, 4 females, weighing 9-20 kg) were infused intravenously with 6.8 mg/kg sodium palmitate. Ten dogs survived the first infusion and were injected again after 3 and 24 hours, and then daily (at rapid doses of 9.7-14.6 mg/kg during three to four minutes) until they died. Blood was drawn from these dogs at various times and examined for platelet counts, platelet adhesiveness, fibrinogen levels, and euglobulin lysis time (see Table 23).

Platelet counts decreased by 40%, 15 minutes following the first infusion, but the counts returned to normal after three hours. After repeated daily infusions, platelets varied within normal limits when counted 24 hours after each infusion. Platelet adhesiveness decreased significantly even at 24 hours after each infusion. Fibrinogen levels did not decrease during any infusion but increased in animals that survived more than seven days after the first infusion. The acceleration of euglobulin lysis time at the end of the first infusion was replaced by a delayed lysis time three hours later.

The authors concluded that these dogs became tolerant to doses of sodium palmitate that would be lethal if given initially. Such perfusions had to be given rapidly in order to cause death.

2. Day, et al. (027) examined surviving male mongrel dogs given an intravenous infusion of a single rapid dose of sodium palmitate (10 mg/kg BW as a 0.1% suspension over a five minute period). The effects of the sodium salts of other fatty acids were also examined. Massive venous and arterial thrombi were found in 8 of 11 necropsied, palmitate-infused dogs. The study indicated that saturated fatty acids, in contrast to unsaturated ones, were thrombogenic.

Table 23

Platelet Counts, Platelet Adhesiveness and Euglobulin Lysis Time in Dogs (024)

Animal	Sex	Platelet Count x 10^3				Adhesiveness %				Euglobulin lysis time/min			
		I[a]	II[b]	III[c]	IV[d]	I	II	III	IV	I	II	III	IV
1	M	301	236	342	---	77	12	0	—	70	60	120	—
2	F	479	246	433	380	84	10	0	12	59	13	110	420
3	M	330	176	240	---	80	30	31	—	40	20	110	—
4	F	715	571	779	530	72	13	19	23	60	60	300	240
5	M	238	201	300	260	22	15	13	16	60	33	140	180
6	F	490	251	456	342	70	41	2	30	50	30	45	60
7	F	765	488	595	420	31	25	19	20	60	60	60	120
8	M	585	200	732	560	55	30	39	19	45	20	50	80
9	M	460	280	470	---	82	20	14	—	90	15	420	—

[a]Initial values.
[b]15 minutes after the beginning of the infusion.
[c]3 hours later.
[d]After daily perfusion for 5 consecutive days, blood harvested 24 hours after the fifth perfusion.

In vitro coagulation tests were performed on blood extracted from the surviving dogs. Results are shown in Table 24. Blood recalcification time was shortened significantly in the dogs receiving saturated (palmitate) fatty acids.

In studying blood lipids, no significant changes were encountered in the sodium palmitate infused dogs, nor in those dogs infused with other fatty acid salts (see Table 25).

The authors stated that the coagulation studies apparently provided *in vitro* evidence that the *in vivo* effect in those animals receiving salts of saturated fatty acids (palmitate) differed from the effect in those receiving salts of unsaturated fatty acids. (For similar results, see Oleic acid, sodium salt, Biological data, Section II, B.)

III. Long-Term Toxicity

No information available.

IV. Special Studies

Mice

Nakahara (090) tested the effects of sodium palmitate on the resistance of mice to transplanted cancer. The sodium palmitate was a 1% solution in distilled water and was administered as a single intraperitoneal injection (see Table 26 for dosages) into mice (young adults, 17-20 g) ten days prior to implantation of Bashford adenocarcinoma no. 63 in the left groin. The results (see Table 26) indicated that sodium palmitate does not display an immunologic effect upon implanted cancer at the levels administered in these experiments. (For a similar experiment, see Oleic acid, sodium salt, Biological Data, Section IV, A.)

Table 24

Mean Changes Noted in Coagulation Tests Following Infusion of Fatty Acid Solution into Dogs (027)

Acid	Coag. Time Glass Min.	Coag. Time Silicone Min.	Recal. Time Sec.	Serum Pro. Time Sec.	Plasma Pro. Time Sec.	Part. Thromb. Time Sec.	Hicks-Pitney TGT Sec.	Stypren Time (1:1) Sec.	Stypren Time (1:4) Sec.	Platelet Count
Oleic C-18:1	−0.187 *16	0.91 11	−1.25 16	−1.3 16	−0.31 15	+0.02 10	+0.06 15	+0.22 15	−0.020 15	−223,400 8
Linolenic C-18:3	0.9 20	−1.8 18	−0.8 19	−10.5 19	−0.05 20	+0.58 18	+1.5 4	+0.5 19	−1.3 20	−135,000 20
Palmitic C-16:0	−0.26 23	−3.6 18	−18.0 17	−20.2 21	−1.6 19	−3.5 12	+0.26 19	−3.6 13	−3.8 16	−197,000 11
Stearic C-18:0	−0.08 12	−1.8 11	−15.0 14	−25.2 14	+0.32 15	+5.0 8	+0.83 12	−3.5 14	−3.8 14	−56,750 8
Oleic-Palmitic	−1.6 21	−3.2 24	−19.2 25	−20.3 23	−1.6 19	+1.66 9	−0.36 11	−3.7 26	−0.4 23	−177,707 14
Oleic-Stearic	−8.0 7	−12.8 5	−30.8 6	−30.5 9	+0.23 9	+2.25 6	+0.66 3	−1.4 9	−0.85 9	−197,000 6
Saline	−0.17 18	−1.2 17	+2.5 16	+0.3 16	+0.08 17	−0.40 16	−0.4 5	+0.43 17	1.73	−84,125 16

*Figure in this position indicates number of paired observations made for the fatty acid concerned.

(Note: In interpreting the results it must be remembered that the injusions were 10 ml/kg into dogs whose blood volume is 92 mg/kg and a slight dilutional effect occurred due to an increase in blood volume, note saline control.)

Table 25

Mean Changes Produced in Lipid Levels by The Intravenous Infusion of Fatty Acid Solutions into Dogs (027)

	Total Chol. mg./100 ml.	*Free Chol. mg./100 ml.*	*Ester. Chol. mg./100 ml.*	*Esters Chol. mg./100 ml.*	*Phospho-Lipids mg./100 ml.*	*Tri-Glycer. mg./100 ml.*	*Total Lipids mg./100 ml.*	*Non-ester. F.A. μEq./L.*
Oleic C-18:1	—8.0 14	—6.3 14	—0.5 14	—3.0 14	—16.2 14	—7.0 14	—26.5 14	—17.0 16
Linoleic C-18:3	—14.6 9	—7.1 10	—7.4 9	—12.6 9	—28.6 9	—6.3 9	—55.2 9	—219.4 10
Palmitic C-16:0	—10.0 17	—2.0 17	—7.5 17	—6.0 16	—27.7 18	+0.125 18	—47.4 18	—19.4 20
Stearic C-18:0	—9.0 13	—8.2 13	—4.4 13	—5.1 13	—30.0 13	—1.7 13	—36.4 13	—146.3 9
Oleic-Palmitic	—7.4 16	—1.8 16	—4.6 16	—10.4 16	—27.4 15	—6.1 15	34.4 16	5.9 16
Oleic-Stearic	—6.0 11	—4.0 11	—3.4 11	—4.7 11	—13.6 11	17.0 11	—31.0 11	—66.5 11
Saline	—15.4 15	—3.4 15	—12.0 15	—19.0 15	—36.0 15	—6.4 15	—50.5 15	—187.4 14

Table 26

Effect of Sodium Palmitate on Resistance to Implanted Cancer ( )

Experiment No.	Amount of sodium palmitate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.[a]				
6	2	8	0 (0.0 per cent).	9	1 (11.1 per cent).
7	4	10	2 (20.0 " ").	9	1 (11.1 " ").
8	6	10	3 (30.0 " ").	9	2 (22.2 " ").
9	8	10	2 (20.0 " ").	9	2 (22.2 " ").
10	10	9	1 (11.1 " ").	8	2 (25.0 " ").
11	12	10	2 (20.0 " ").	8	2 (25.0 " ").
Average.....		57	10 (17.5 per cent).	52	10 (19.2 per cent).

[a]mg = 0.1 ml of 1% solution

Table 26

Effect of Sodium Palmitate on Resistance to Implanted Cancer (090)

Experiment No.	Amount of Sodium Palmitate	Treated Mice		Controls	
		No.	Resistant Mice	No.	Resistant Mice
	mg[a]				
6	2	8	0 (0.0%)	9	1 (11.1%)
7	4	10	2 (20.0%)	9	1 (11.1%)
8	6	10	3 (30.0%)	9	2 (22.2%)
9	8	10	2 (20.0%)	9	2 (22.2%)
10	10	9	1 (11.1%)	8	2 (25.0%)
11	12	10	2 (20.0%)	8	2 (25.0%)
Average.....		57	10 (17.5%)	52	10 (19.2%)

mg[a] = 0.1 ml of 1% solution.

BIOCHEMICAL ASPECTS

I. Breakdown

No information regarding cases of spontaneous breakdown of sodium palmitate in food under conditions of storage, processing or cooking has been found in current literature.

II. Absorption and Distribution

Introduction

Ingested neutral fats (triglycerides) are normally hydrolyzed and absorbed by the intestine as monoglyceride and free fatty acid. Following absorption, triglycerides are reformed in the intestinal mucosa and incorporated into chylomicrons or lipoprotein (very low density) for transportation throughout the organism (026).

In cases where there is an excess of free fatty acid, i.e., where the ratio of absorbed fatty acid to monoglyceride is greater than 2:1, as would be the case in ingesting the free fatty acid, the fatty acid may (after activation with coenzyme A) be combined with alpha-glycerophosphate to form a triglyceride and be incorporated into chylomicrons as above (026).

Rats

1. Bloom et al. (011) administered palmitic acid-^{14}C in 0.5 ml corn oil or tripalmitin-^{14}C to male rats (Long-Evans strain) with cannulated thoracic ducts by intragastric intubation. Samples were collected from the thoracic duct and intestinal lacteals for observation. At the end of the test period, the contents of the stomach, large and small intestines, cecum, and feces of each rat were examined.

In 19-24 hours, 12 of the fourteen rats absorbed from 81-95% of the

administered labeled palmitic acid (see Table 27,28). From 70 to 92% of the absorbed palmitic acid-^{14}C was recovered as fatty acid-^{14}C from thoracic duct lymph in 9 of 10 rats, whereas 69-84% of the absorbed palmitic acid-^{14}C was recovered as fatty acid-^{14}C from intestinal lymph of four rats.

Table 27

Recovery of Enterally Administered Labeled Palmitic Acid in Thoracic Duct Lymph (011)

The palmitic acid used was labeled in the carboxyl position with C^{14}. It was dissolved in 0.5 cc of corn oil at body temperature and administered by intragastric intubation. Each rat received approximately 4 mg of labeled palmitic acid containing 1 x 10^5 counts per minute per mg.

Rat No	Weight	Total lymph collected				Form of labeled palmitic acid administered	Per cent of administered palmitic acid-C^{14} recovered		Per cent of administered palmitic acid-C^{14} absorbed	Per cent of absorbed palmitic acid-C^{14} recovered	
		Before fat administration		After fat administration			In stomach and small intestine contents as lipide-C^{14}	In cecum and large intestine contents and feces as lipide-C^{14}		In lymph as fatty acid-C^{14}	In liver as lipide-C^{14}
	gm.	hrs.	cc.	hrs.	cc.						
1	200	52	108	20	43	Tripalmitin	2.3	17.2	80.5	85.7	0.34
2	238	28	14	20	36	"	0.5	51.8	47.7	58.3	0.41
3	185	23	42	23	94	"		7.3*	92.7	70.7	0.19
4	287	24.5	38	24	91	"		5.0*	95.0	73.0	0.19
5	200	53	204	19	98	Palmitic acid	1.4	9.8	88.8	70.0	0.35
6	310	27	28	21	101	" "	0.7	6.7	92.6	76.4	0.11
7	386	27.5	49	21	118	" "	2.3	14.5	83.2	78.5	0.26
8	201	21	58	19	78	" "		13.5*	86.5	69.8	0.23
9	183	23	65	19	96	" "		15.5*	84.5	79.7	0.20
10	260	24	12	21	45	" "	0.2	14.2	85.6	91.8	0.25

* The entire gastrointestinal contents were combined for analysis.

Table 28

Recovery of Enterally Administered Labeled Palmitic Acid in Intestinal Lymph (011)

The palmitic acid used was labeled in the carboxyl position with C^{14}. It was dissolved in 0.5 cc of corn oil at body temperature and administered by intragastric intubation. Each rat received approximately 4 mg of labeled palmitic acid containing 1×10^5 counts per minute per mg.

Rat No.	Weight	Total lymph collected: Before fat administration		Total lymph collected: After fat administration		Form of labeled palmitic acid administered	Per cent of administered palmitic acid-C^{14} recovered: In stomach and small intestine contents as lipide-C^{14}	Per cent of administered palmitic acid-C^{14} recovered: In cecum and large intestine contents and feces as lipide-C^{14}	Per cent of administered palmitic acid-C^{14} absorbed	Per cent of absorbed palmitic acid-C^{14} recovered: In lymph as fatty acid-C^{14}	Per cent of absorbed palmitic acid-C^{14} recovered: In liver as lipide-C^{14}
	gm.	hrs.	cc.	hrs.	cc.						
20	260	28	20	24	25	Palmitic acid	0.6	6.6	92.8	83.8	0.17
21	250	23	40	24	46	" "	0.2	64.5	35.3	83.2	0.39
22	350	22	15	25	52	" "	0.3	13.9	85.8	68.7	1.1
23	290	20	5	24	24	" "	3.9	6.1	90.0	74.8	0.58

In a later study, Bloom et al. (010) attempted to determine the participation of lymph phospholipids in transporting absorbed fatty acids from the small intestine to the plasma. Palmitic acid-^{14}C was administered as described in the above experiment. Lymph was collected. Lipids extracted from each lymph sample were analyzed for contents of fatty acid-^{14}C, phospholipid fatty acids, total fatty acids and cholesterol. 96% of the fatty acid-^{14}C lymph was present in non-phospholipid form, thus demonstrating the unimportance of phospholipids as vehicles for fatty acid transport.

2. Herting *et al*. (055) fed weanling Holtzman rats (3 males and 3 females) with palmitic acid and determined the acid's absorbability for a one week period during the 12th and 13th weeks of the experiment. The results are tabulated in Table 29.

Table 29

Palmitic Acid Absorbability (055)

Fat	Sex	Food consumption (g/rat)	Saturated fatty acid balance consumed (g)	excreted (g)	absorbed (g)	absorbability %
Palmitic acid	M	85.5	42.8	18.0	24.8	58.0
	F	91.7	45.8	18.4	27.4	59.8

III. Metabolism and Excretion

Introduction

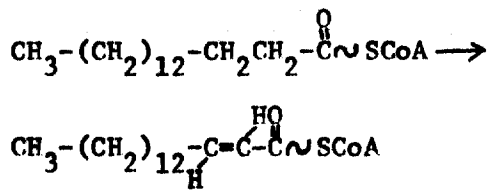
The major pathway whereby palmitic acid is catabolized in the animal body is beta-oxidation, consisting primarily of five repeated steps occurring in the cell mitochondria (081,098):

a. Activation-- A long chain fatty acid thiokinase [fatty acid: Co A ligase (AMP)] in the presence of ATP transforms the free palmitic acid to a palmitoyl coenzyme A derivative.

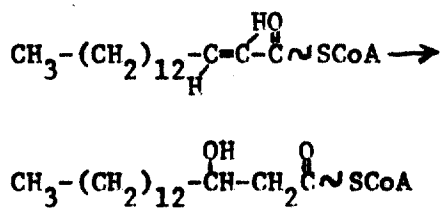
$$CH_3-(CH_2)_{12}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-OH + ATP + CoA-SH \longrightarrow$$

$$CH_3-(CH_2)_{12}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}\sim SCoA + AMP = PPi$$

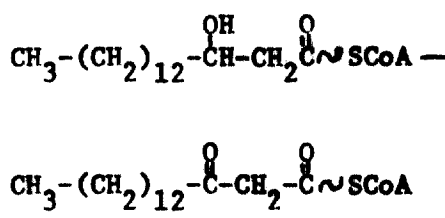
b. Dehydrogenation-- The appropriate fatty acyl Co A dehydrogenase desaturates the palmitoyl Co A derivative in the alpha and beta positions, resulting in a <u>trans</u> configuration at this double bond.



c. Hydration-- One molecule of water is added across the double bond in the presence of enolyhydratase (3-hydroxy-Co A hydrolase) forming the beta-hydroxypalmitoyl Co A derivative of L configuration.



d. Oxidation (2nd dehydrogenation)— L-3-hydroxy fatty acyl Co A dehydrogenase oxidizes the hydroxyl group of the beta-hydroxy palmitoyl derivative to a keto group. The coenzyme NAD^+ is reduced to NADH.



e. Thiolytic cleavage-- Beta-ketothiolase breaks the beta-keto derivative to form acetyl Co A and adds one molecule of coenzyme A back to the fatty acid, forming an activated fatty acid which is 2 carbons less than the original palmitic acid.

$$CH_3-(CH_2)_{12}-\overset{O}{\overset{\|}{C}}-CH_2-\overset{O}{\overset{\|}{C}}\sim SCoA + CoASH \longrightarrow$$

$$CH_3-(CH_2)_{12}-\overset{O}{\overset{\|}{C}}\sim SCoA + CH_3\overset{O}{\overset{\|}{C}}\sim SCoA$$

The fatty acid repeats the cycle b, c, d, e, until the entire chain is consumed, resulting in 8 molecules of acetyl Co A which may condense with oxaloacetic acid and be oxidized through the Krebs carboxylic acid cycle, and eventually leave the body as exhaled CO_2.

Rats

Baker and Schotz (003) studied palmitate-1-^{14}C metabolism in fasted rats. Male Sprague-Dawley rats (160-200 g) fasted for 20 hours and were intravenously injected with 3 μc of palmitate-1-^{14}C complexed to 0.30 ml of fresh rat serum. Rats were sacrificed at various intervals and their carcasses and blood analyzed. The results are tabulated in Table 30.

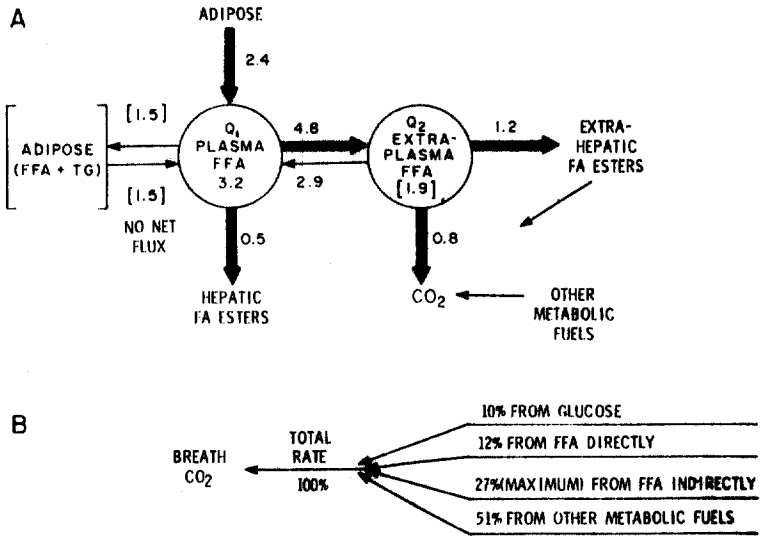


Fig. 8. Plasma free fatty acid exchange, esterification and oxidation (003). Rates are expressed as μmoles of fatty acid/min per 100 g body weight. Pool sizes are given as μmoles of fatty acid/100 g body weight. Pool size Q_2 is shown in brackets to indicate that the calculated value is a minimum estimate. The lower part of the figure, B, relates the estimated rates of FFA oxidation by direct and indirect pathways to the total rate of CO_2 oxidation.

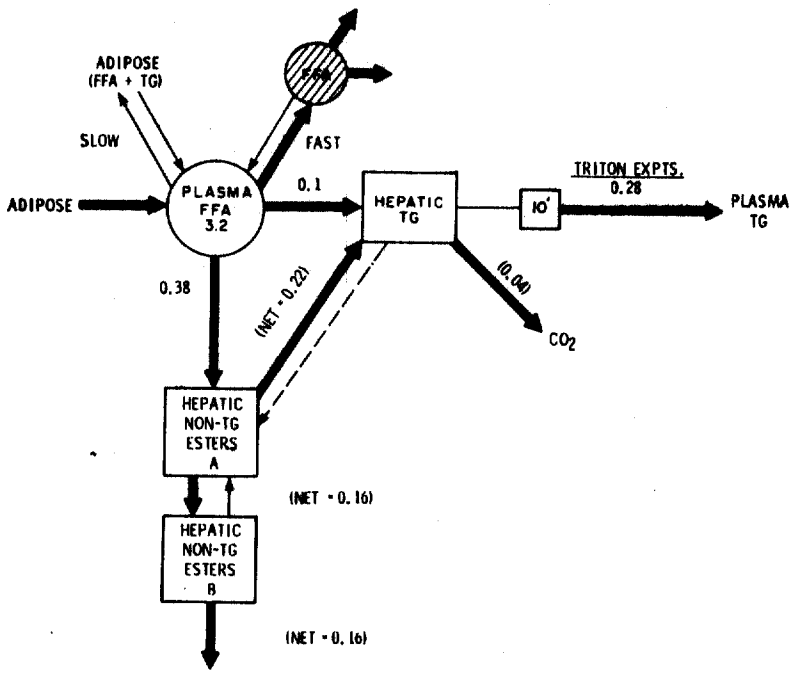


Fig. 9. Hepatic triglyceride formation (003).
Rates of hepatic TG formation by direct and relatively indirect pathways. The rates and pool sizes are expressed in the units given in figure 8. The rate of TG secretion is taken from separate experiments and is a minimum estimate. The rate of direct hepatic TG formation from plasma FFA was found to be only a fraction of the total rate of TG formation. The remainder of the required TGFA (0.22 μmoles/min. of TGFA/100 g body wt) was assumed to come from non-TG esters which were formed from plasma FFA at a rate sufficient to supply the required net flow of FA.

Table 30

Palmitate-1-^{14}C Metabolism (003)

Substance	0	2 sec.	Percent of injected ^{14}C 1 min.	10 min.	1 hr.	3 hr.	6 hr.
FFA (total body)	100	91	44	10	< 1	< 1	< 1
Other lipid (total body)	---	8	32	57	57	42	30
Total lipid	---	99	76	67	57	42	30
Exhaled CO_2	---	0	0.5	8	38	57	65
Missing	---	1	24	25	5	1	5

At one, three and six hours, 30-57% of the injected ^{14}C was recovered as triglycerides and phospholipids of hepatic and extrahepatic tissues and the rest as expired CO_2. At one minute, all injected ^{14}C could be recovered as fatty acids if the rats were saponified for one hour in alkaline ethanol at 100° C, indicating that the missing ^{14}C was in lipid form. At 10 minutes, 13% of the missing ^{14}C was recovered as labeled CO_2, indicating that the palmitate had been converted to nonlipid compounds.

Using a multicompartmental model and a digital computer, the rates were calculated of:

a. plasma free fatty acid exchange, esterification, and oxidation (Figure 8),

b. hepatic triglyceride formation (Figure 9) and,

c. extrahepatic triglyceride and non-triglyceride formation (Figure 10).

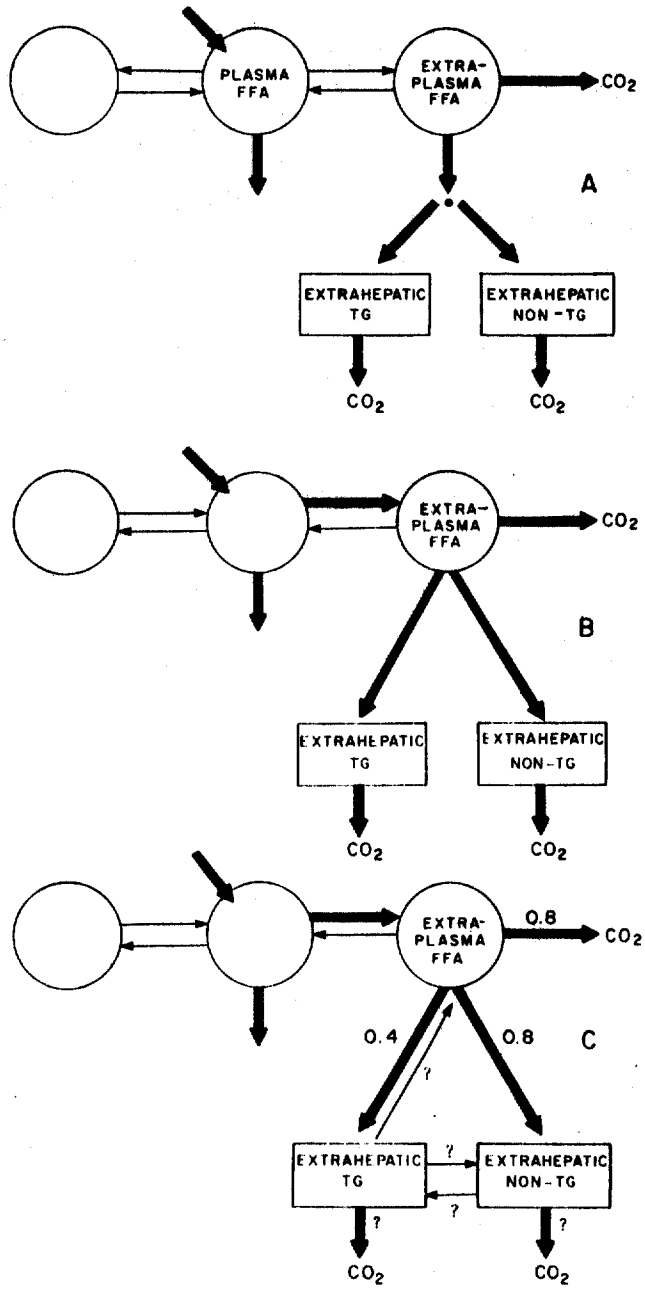


Fig. 10. Extrahepatic triglyceride and non-triglyceride formation (003). Estimation of rates of TG and non-TG formation from fatty acids in extrahepatic tissues. A shows that part of the initial model which was to have served as a basis for calculating net rates of esterification after simplification to model B. However, the data indicated that a more complex model such as that shown in C may be required to describe the interrelations between extraplasma FFA and extrahepatic TG and non-TG esters. The data obtained could not define rates of flow shown by question marks. However, if it is assumed that no net extraplasma FFA is formed from TG or non-TG esters in nonadipose, extrahepatic tissues, then the rates of direct esterification shown in C may be calculated from fractional rate constants and the pool sizes shown in Fig. 8. Units are given in Fig. 9.

IV. Effects on Enzymes and Other Biochemical Parameters

In vitro

1. Because the sodium salts of long chain fatty acids were reported to cause massive thrombi in dogs, Surgala, et al. (121) undertook a study of the fibrinolytic activity of the salts of fatty acids, with regard to the influence of saturation.

To Petri dishes prepared with 10 ml bovine fibrinogen (2.5 mg/ml) and 0.5 ml bovine thrombin (50 NIH units/ml) in sodium borate buffer pH 7.7 was added 0.02 ml of 2-fold dilutions of the salts of fatty acids. The lowest concentration causing liquefaction sufficient for complete perforation of the fibrin film after incubation at 37° C for at least four hours was taken as the end-point.

Table 31 presents the results. The authors suggested that the saturated fatty acids of which palmitate is representative were less fibrinolytic than the unsaturated fatty acid salts. (For a similar experiment, see oleic acid, sodium salt, Biochemical Aspects, Section IV, In vitro, 1.)

2. Kerr, et al. (065) tested the effect of fatty acids on platelet aggregation. Approximately 10 mg of the sodium salt of palmitic acid were powdered and slowly added to 1.0 ml of purified lecithin giving a palmitate-lecithin sol of 0.0035 M. 0.5 ml of the sol was added to 1.5 ml of platelet-rich plasma (blood drawn from humans), and the optical density measured for platelet aggregation.

The palmitate-lecithin sol caused platelet aggregation which tended to be transient, comparable with oleate-lecithin and myristate-lecithin sols. Figures 11a and b show the results of the palmitate experiment compared to other saturated and unsaturated fatty acid sols. (For a similar experiment, see

Table 31

Influence of Unsaturation on Fibrinolytic Activity (121)

Fatty Acid		Active concentration, mM — Test No.[a] 1	2	3	4	5
Palmitate	C16:0		5	.3	.3	
Palmitoleate	C16:1		.5	.3	.3	
Stearate	C18:0		.6	1	1	
Oleate	C18:1	.2	.06	.2	.08	
Elaidate	C18:1		.06	.2	.2	
Arachidate	C20:0		1	5	1	
Eicosenoate	C20:1					.08
Behenate	C22:0		>10	>10	>10	
Erucate	C22:1		.03	.02	.04	
Lignocerate	C24:0					>10
Nervonate	C24:1					.08

a = Test 1: sodium salt
Test 2-5: potassium salt

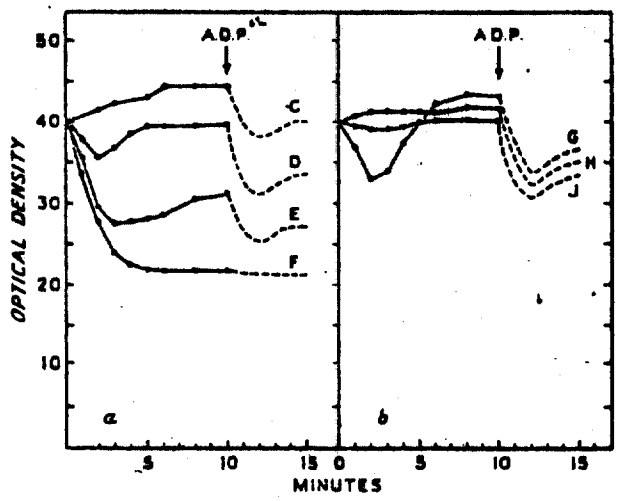


Fig. 11a. Effect of various sols of lecithin with saturated fatty acids on platelet-rich plasma. Means of 8 patients. (068)

C. -- No fatty acid.
D. -- Myristate (14-carbon).
E. -- Palmitate (16-carbon).
F. -- Stearate (18-carbon).

Fig. 11b. Effect of various sols of lecithin with unsaturated fatty acids on platelet-rich plasma. Means of 8 patients.

G. -- Oleate (18-carbon).
H. -- Linolenate (18:3-carbon).
J. -- Linoleate (18:2-carbon).

[a]A.D.P (adenosine diphosphate), 1 or 2 ug in 0.01 ml barbiturate buffered saline added to asses viability of the platelets.

Oleic acid, sodium salt, Biochemical Aspects, In vitro, 2.)

3. Goodman (042) studied the interaction of human erythrocytes with sodium palmitate by measuring the distribution of the palmitate between erythrocytes and serum albumin. Increasing quantities of sodium palmitate were equilibrated in a mixture of equal volumes of red cells (orghosts) and serum albumin in isotonic phosphate buffer-- NaCl. The results showed that there were 5.3×10^{-17} moles of sodium palmitate/ erythrocyte (for detailed calculations, see original paper). The binding sites were located on the erythrocyte ghosts, suggesting that the initial steps in plasma unesterified fatty acid metabolism involved the transfer of unesterified fatty acids from serum albumin to receptor binding sites on cell surfaces. (For a similar experiment, see oleic acid, sodium salt, Biochemical Section IV, In vitro, 7.)

In vivo

Rats

Ehrenstein and Corey (031) used young male, castrated white rats, weighing 50-80 g, to assay the activating effect of palmitic acid or sodium bipalmitate ($C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$) on testosterone. Testosterone, with and without palmitic acid or sodium bipalmitate, was injected subcutaneously into rats for nine days. The animals were sacrificed and the development of their seminal vesicles and prostrate glands was examined. Results tabulated in Table 32, showed that palmitic acid and its acid sodium salt increased the effect of testosterone on the accessory sex organs of the male rat.

The authors noted that rather low doses (up to 25 mg daily) of the salt proved to be more effective than the same dose of the free acids. Activation with the acid sodium salt could not, however, be increased beyond that obtained with the 50 mg daily dosage.

Table 32

Activating Effect of Palmitic Acid or Sodium Bipalmitate on Testosterone (031)

No. of rats	Concentration (mg) and kind of activator	Avg. body wt. at killing (g)	Avg. wt. Seminal vesicles (mg)	Avg. wt. Prostate (mg)	Seminal vesicles (% of BW)	Prostate (% of BW)
4	25 Palmitic acid + T[a]	145	51	46	0.034	0.031
4	50 Palmitic acid + T	156	154	86	0.100	0.055
4	10 Na bipalmitate + T	146	92	66	0.060	0.045
6	25 Na bipalmitate + T	135	139	75	0.104	0.055
6	50 Na bipalmitate + T	126	185	96	0.147	0.075
5	No injection	150	205	110	0.134	0.072
4	50 T	140	25	21	0.018	0.016

[a]T = testosterone.

V. Drug Interaction

Hoshino, et al. (057) studied the effect of sodium palmitate on leucomycin absorption, in vitro, using the everted sac of rat intestine. At 125-250 mμg/ml, sodium palmitate increased the absorption of leucomycin from rat mucous membrane.

VI. Consumer Exposure

As discussed earlier, (see Chemical Information, Section VIII) the sodium salts of oleic and palmitic acids are produced by the alkaline hydrolysis of neutral fats (triglycerides). These salts may be used as food additives when applied as binders, emulsifiers, or anti-caking agents provided they are prepared from fatty acids which conform to food grade specifications.

No information regarding production levels, annual poundage or possible daily intake is available. The Joint FAO/WHO Expert Committee on Food Additives (061) has set no limit on the acceptable daily intake of sodium palmitate, provided the contribution of cations does not exceed the normal body load. Percentage use in food categories was not given in the NAS/NRC tables; however, the WITCO Chemical Corporation (141) suggested use of sodium palmitate as a thickener and emulsifier for aqueous systems at 1-5% levels.

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VOLUME 2

GRAS MONOGRAPH SERIES

SODIUM SALTS OF FATTY ACIDS

(COPIES OF ARTICLES REFERENCED IN TEXT)

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cold H_2O. Det. both K and Na in filtrate as in 6.015 and 6.020 or 6.016–6.019.

MINERAL OILS

Unsulfonated Residue (*26*)—Official, Final Action

4.099 REAGENT

Fuming 38N sulfuric acid.—In tared g-s. bottle (2.5 L acid bottle is convenient) mix fuming H_2SO_4 (free from N oxides) (A) with H_2SO_4 (B) to obtain mixed acid (C), contg slightly >82.38% total SO_3. Depending on strength of fuming acid available, use following proportions of 2 acids: 100 parts A (15–20% free SO_3) to 50 parts B; 100 parts A (20–30% free SO_3) to 75 parts B; and 100 parts A (50% free SO_3) to 140 parts B. Mix thoroly (considerable heat is generated), let cool, and again weigh to det. quantity mixed acid obtained. Det. exact strength of mixed acid (C) and also of reserve supply of acid (B) as follows:

Pour ca 50 ml into small beaker and fill ca 10 ml weighing bulb or pipet by slight suction, wiping off outside of bulb with moist, then with dry, cloth. Weigh on analytical balance and let acid flow slowly down sides of neck of 1 L vol. flask into ca 200 ml cold H_2O. (The sizes of bulb and flask give final soln ca 0.5*N*.) When bulb has drained, wash all traces of acid into flask, taking precautions against loss of SO_3 fumes. Dil. to vol. and titr. from buret with std alkali, using same indicator as used in stdzg. Calc. SO_3 content of both acids, and add calcd quantity of reserve acid (B) to quantity of mixed acid (C) on hand to bring C to 82.38% total SO_3 (equiv. to 100.92% H_2SO_4). After adding required quantity of B, again analyze mixed acid to make certain it is of proper concn (±0.15% H_2SO_4). Keep acid in small bottles or in special dispenser bottle (*27*) to prevent absorption of H_2O from air.

4.100 DETERMINATION

Pipet 5 ml sample into 6" Babcock cream bottle, 15.087(a), either 9 g 50% or 18 g 30% type. To reduce viscosity of heavy oils, warm pipet after initial drainage by passing it several times thru flame; then drain thoroly. If greater accuracy is desired, weigh measured charge and calc. its exact vol. from wt and sp. gr. Slowly add 20 ml of the 38*N* H_2SO_4, gently shaking or rotating bottle and taking care that temp. does not H_2SO_4 until oil is in graduated neck. Centrifuge 5 min. (or longer if necessary to obtain constant vol. of oil) at 1200–1500 rpm. Read vol. of unsulfonated residue from graduations on neck of bottle and, to convert to ml, multiply reading from 9 g 50% bottle by 0.1 and reading from 18 g 30% bottle by 0.2. From result obtained calc. % by vol. unsulfonated residue.

MINERAL OIL-SOAP EMULSIONS

Water (*28*)

4.101 *Toluene Distillation Method—Official, Final Action*

Weigh ca 25 g sample and proceed as in 4.097, except use less rosin.

4.102 Total Oil (*29*)—Official, Final Action

Weigh ca 10 g sample into Babcock cream bottle, 15.087(a). Dil. with ca 10 ml hot H_2O and add 5–10 ml H_2SO_4 (1+1). Heat in hot H_2O bath ca 5 min. to hasten sepn of oil, add enough satd NaCl soln to bring oil layer within graduated neck of bottle, whirl at 1200 rpm 5 min., and let cool. Read vol. of oil layer, det. density, and from these values calc. wt and %. From this % value deduct % fatty acids (and phenols if present), detd separately, to obtain % oil.

4.103 Soap (*28*)—Official, Final Action

(Error will result if apparent molecular wt of fatty acids varies appreciably from that of oleic acid.)

Weigh 20 g sample into separator, add 60 ml petr. ether, and ext. mixt. once with 20 ml and 4 times with 10 ml 50% alcohol. Break emulsion if necessary by letting 1 or 2 ml 20% NaOH soln run down wall of separator. Then gently swirl separator and let stand few min. Drain alc. layers and wash successively thru petr. ether contained in 2 other separators. Combine alc. exts in beaker and evap. on steam bath to remove alcohol. Dissolve residue in ca 100 ml H_2O made alk. with NaOH. Transfer to separator, acidify with HCl or H_2SO_4, ext. 3 times with ether, and wash ether exts twice with H_2O. Combine ether exts, evap. in weighed beaker on steam bath, and weigh as fatty acids. From wt fatty acids calc. % soap in sample as Na or K oleate.

4.104 Unsulfonated Residues—Official, Final Action

Quantitative aspects of free fatty acid metabolism in the fasted rat

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ABSTRACT Palmitate-1-^{14}C was injected intravenously into unanesthetized, fasted rats. Disappearance of tracer from plasma free fatty acids was studied. A large component of free fatty acid (FFA) recycling was directly demonstrated by reinjection experiments. The latter studies also indicated the existence of an unidentified, rapidly turning over polar lipid in plasma which was synthesized from palmitate-^{14}C. The appearance of ^{14}C in hepatic and extrahepatic triglycerides, in other esters, and in respired CO_2 was also followed. The data were analyzed using a multicompartmental model and a digital computer.

Only a small fraction of the triglycerides formed in liver was derived directly from plasma free fatty acids. The major portion of net triglyceride formation appeared to be by way of an intermediate nontriglyceride ester pool which turned over relatively slowly compared to plasma free fatty acids. Initial approximations are as follows (μmoles of fatty acid per min per 100 g body weight): net free fatty acid mobilization (irreversible disposal) = 2.4; hepatic triglyceride formation directly from plasma free fatty acid = 0.1; total hepatic lipid formation from plasma free fatty acids = 0.5; oxidation of free fatty acids to CO_2 = 0.8; percentage of respired CO_2 from direct oxidation of fatty acids = 12%; extrahepatic triglyceride formation directly from fatty acids = 0.4; total extrahepatic lipid formed directly from fatty acids = 1.2.

KEY WORDS bicarbonate-^{14}C · CO_2 · extrahepatic · fasted rats · fatty acid oxidation · free fatty acid turnover · liver · irreversible disposal · lipid esters · multicompartmental analysis · palmitate-1-^{14}C · recycling · triglycerides · triglyceride secretion

In an earlier study of hepatic TG secretion in fasted rats, we estimated that the TG turnover rate in liver was several times greater than the rate of hepatic TG synthesis from plasma FFA (1). This conclusion was difficult to reconcile with current concepts of FA and TG metabolism in fasted animals, according to which virtually all newly synthesized hepatic TGFA is derived from circulating FFA (2–4). Since hepatic lipogenesis is depressed in the latter condition, newly synthesized hepatic TGFA cannot be synthesized rapidly from nonlipid precursors. Therefore, if TG is turned over in the liver faster than it is synthesized from FFA, the TG must be derived from some other lipid pool such as PL. This conclusion, however, was based upon experiments which were not specifically designed to measure the rate of FFA conversion to liver TG (1).

In the present study, we have reexamined this problem using an experimental approach which focused upon plasma FFA turnover at early times after palmitate-1-^{14}C injection and upon the initial rates of incorporation of plasma FFA into hepatic and extrahepatic TG.

METHODS

Male Sprague-Dawley rats (160–200 g) were deprived of food for 20 hr, but given free access to drinking water. 3 μc of palmitate-1-^{14}C (31 μc/μmole; New England Nuclear Corp., Lot No. 31-238-7) was complexed to 0.30 ml of freshly prepared rat serum (5) and injected into the tail vein of unanesthetized rats. At various time intervals after the injection, a few rats were stunned with a blow and quickly immersed in liquid nitrogen. Other rats were decapitated; the blood was collected in plastic tubes containing solid sodium oxalate and immediately chilled in an ice bath. Simultaneously with the blood withdrawal, the abdominal cavity was ex-

Abbreviations: FFA, free fatty acid(s); TG, triglyceride(s); TGFA, triglyceride fatty acid; PL, phospholipid(s).

posed and a piece of liver tissue was quickly extirpated, immersed in liquid nitrogen, weighed, and extracted for total lipids with chloroform–methanol 2:1 (6). The remaining liver was also excised and weighed to obtain total liver weight. The carcasses were quickly frozen in liquid nitrogen and either extracted immediately or stored at −16°C for 3 days prior to extraction.

The plasma samples were extracted for lipids as described by Sperry (7). The frozen whole rats and the carcasses were crushed and then extracted with 1800 ml of chloroform–methanol 2:1 in a 1 gallon Waring Blendor. Prior to extraction extreme care was taken to keep the carcasses frozen. The homogenates were diluted to 2000 ml and aliquots of the filtered material washed with $^1/_5$ their volume of water. The water-washed extract was dried under a stream of nitrogen and stored at −16°C in a chloroform solution. The chloroform–methanol extracts of plasma and liver were washed, dried, and stored in a similar manner. The chloroform–methanol aliquots of each of the samples for each time point were pooled, except for the 2-sec and 1-min groups, which were determined individually.

Aliquots of each of the lipid extracts were fractionated by thin-layer chromatography on silica gel (8). The various fractions were detected by exposure to iodine vapors, removed from the plates, and assayed for ^{14}C as previously described (8).

Recycling Experiment

Six unanesthetized rats were fasted and then injected with approximately 15 μc of palmitic acid-1-^{14}C complexed to bovine serum albumin. Exactly 5 min after injection of the labeled material, each rat was decapitated and blood was collected. Serum from each of the rats was pooled and an aliquot was set aside for subsequent assay. 0.50 ml of the labeled serum, which was subsequently found to contain 2920 cpm of polar lipids and 6530 cpm of FFA, was then injected into the tail veins of 14 other fasted, unanesthetized rats. 1, 2, and 5 min after the injection the animals were decapitated and the blood was collected into chilled tubes containing a drop of sodium heparin (10 mg). Plasma lipids and an aliquot of the injected serum were extracted with chloroform–methanol 2:1, washed, dried, and fractionated by thin-layer chromatography (8). Approximate midpoint R_f values were as follows: phospholipids, 0; FFA, 0.65; Tg, 0.8; other lipids, rest of plate. Fractions were scraped from the plates and assayed for ^{14}C (8). Plasma FFA concentrations of six separate rats, which had been injected with saline 5 min before they were decapitated, were determined by titration of unwashed isooctane extracts (9).

Respired CO_2

$^{14}CO_2$ was collected in 50 ml of 1 N NaOH at nine different 4 min intervals, spaced over 6 hr, from each of four rats injected with tracer palmitate-1-^{14}C (3 μc complexed to 0.30 ml of rat serum) and from each of four rats injected with tracer sodium bicarbonate-^{14}C (5 μc in 0.50 ml). The same rats were used for measuring the rate of CO_2 expiration. Aliquots were taken for assay of radioactivity in each sample of alkaline sodium carbonate (10). At seven of the time intervals in each experiment, aliquots obtained from individual rats were pooled thus giving a total of 14 samples from eight rats. The carbonate was precipitated with $BaCl_2$ and analyzed titrimetrically.

RESULTS

Plasma FFA

As we noted previously in mineral oil- and CCl_4-fed fasted rats (11), the disappearance curve of FFA^{14}C from the circulation was a complex exponential function (Fig. 1). 1 min after intravenous injection of palmitate-1-^{14}C 30% of the injected ^{14}C remained in plasma; only 1% remained after 5 min.

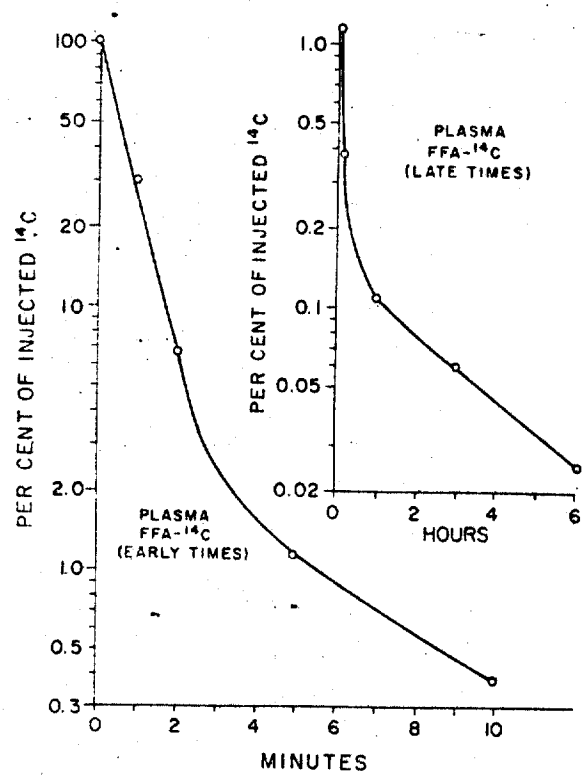


FIG. 1. Percentage of injected palmitate-1-^{14}C in plasma FFA after a single intravenous injection of palmitate-1-^{14}C complexed to rat serum into unanesthetized, fasted rats. Each value represents a pooled aliquot of four rats, except at 1 min when plasmas from four individual rats were analyzed. At 1 min the mean (±SD) value was 30 ± 4.8% of the injected ^{14}C as plasma FFA. Data are plotted on a semilogarithmic scale.

The plasma FFA concentration of a separate group of six rats, fasted overnight, was 0.79 ± 0.12 μmole/ml. The plasma volume of these rats was found to be approximately 4% of body weight (11). From these values, the plasma FFA pool size was estimated to be 3.2 μmoles of FFA per 100 g body weight.

Recycling of FFA

The observations that FFA disappeared from the circulation at an ever-decreasing rate and that a fraction of 1% of the dose persisted in the plasma beyond the 5th min (Fig. 1) suggested that either there was extensive recycling of FFA back into the circulation or that a minor, slowly turning over, labeled substance had been present in the injected dose. The small fraction of ^{14}C present in plasma at 5 min was shown by reinjection into recipient rats to be a rapidly turning over substance (Fig. 2). Therefore, the apparently slow disappearance of FFA-^{14}C from the circulation after the first 5 min of the experiment (Fig. 1) must represent continuous recycling of labeled FFA into plasma. Although no TG-^{14}C is usually found in plasma 5 min after an intravenous injection of palmitate-^{14}C (2, 8, 12, 13) a ^{14}C-labeled polar lipid was found in the PL fraction in the serum of donor rats 5 min after injection of labeled palmitate.[1] The PL fraction contained 45% as much radioactivity as the FFA fraction and, as shown in Fig. 2, seemed to disappear at a rapid rate which, together with its early appearance in plasma of the donor rats, suggests that it is a rapidly turning over PL which may be of considerable interest.

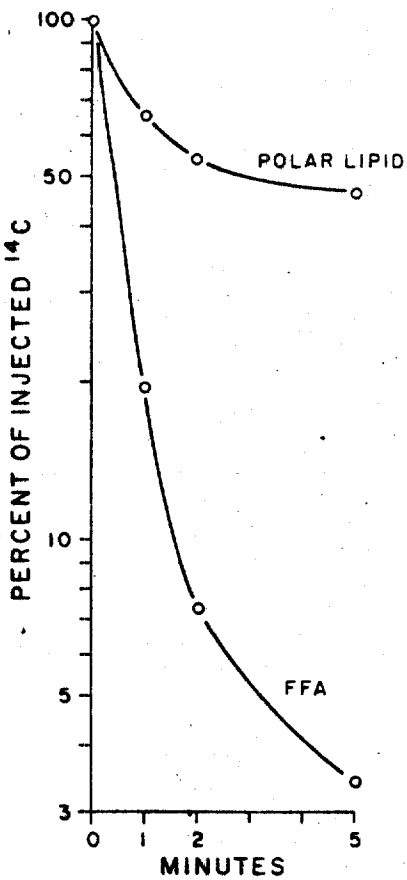


FIG. 2. Disappearance of ^{14}C from plasma FFA and polar lipids after reinjection of plasma obtained 5 min after a single injection of palmitate-1-^{14}C. The fractional SD for the values of plasma FFA at the three times studied were ±0.28, ±0.073, and ±0.25 (n = 5, 4, and 5, respectively). Data are plotted on a semilogarithmic scale.

TABLE 1 BALANCE OF INJECTED ^{14}C

Substance	Per Cent of Injected ^{14}C						
	0	2 sec	1 min	10 min	1 hr	3 hr	6 hr
FFA (total body)	100	91	44	10	<1	<1	<1
Other lipid (total body)	—	8	32	57	57	42	30
Total lipid	—	99	76	67	57	42	30
Exhaled CO_2	—	0	0.5	8	38	57	65
Missing	—	1	*21*	*25*	5	1	5

Disposition of FFA-1-^{14}C

An attempt was made to measure the chemical form and distribution of ^{14}C that had left the plasma. As shown in Table 1, almost all radioactivity could be accounted for at 2 sec, 1 hr, 3 hr, and 6 hr after the injection. At 2 sec, most of the radioactivity was found to be in the form of FFA and was assumed to be still in the form of albumin-bound palmitate-1-^{14}C in plasma. At 1 hr, 3 hr, and 6 hr, 30-57% of the injected ^{14}C was recovered as TG and PL of hepatic and extrahepatic tissues, and the rest as expired CO_2. However, at 1 and 10 min after injection, a large fraction of ^{14}C was neither present as expired CO_2 nor as chloroform-methanol extractable material (Table 1).

The inability to account for ^{14}C in the lipid extract of the frozen rat 1 min after injection of palmitate-^{14}C had also been observed in separate, preliminary experiments.[2] In one instance 94% of the injected dose was recovered in

[1] Dr. B. Lombardi, University of Pittsburgh School of Medicine, Pittsburgh, Pa., informed us prior to our experiment (personal communication) that he has found that the specific activity of plasma total phospholipids reached a maximum at 5 min after intravenous injection of palmitate-^{14}C in rats and declined rather rapidly thereafter.

[2] M. C. Schotz, N. Baker, and M. N. Chavez found in a separate study of fasted and glucose-fed rats that 1 min after injection of palmitate-1-^{14}C all of the injected ^{14}C was recovered in a chloroform-methanol extract of frozen animals. Comparison of these experiments with the present data suggested that our missing activity was probably unesterified fatty acid in tissues. We have not established the cause of this discrepancy.

TABLE 2 Recovery of ^{14}C from an Alkaline Ethanol Extract of Frozen Rats 1 and 10 Min after Injection of Palmitate-1-^{14}C

Expt.	Analysis	2 Sec	1 Min	2 Min	10 Min
			%		
I	Total lipid fatty acid*	— (n = 4)	100 ± 17 (n = 4)	83 ± 10 (n = 4)	46 ± 5 (n = 4)
II	Body bicarbonate†	0 (n = 3)	1.5 ± 0.16 (n = 4)	4.9 ± 0.87 (n = 4)	13.1 ± 3.4 (n = 4)

Values are % of injected ^{14}C, mean ± SD.

* After saponification. In a separate experiment, the recovery of ^{14}C in the whole homogenate (before saponification) at 1 min (n = 3) was not significantly different from that at 2 sec (n = 3) after injection: 4.5 (range, 4.2–4.8) × 10^5 cpm compared with 4.7 (range 4.6–4.7) × 10^5 cpm, respectively.

† Each rat was homogenized in 2 liters of an ethanolic solution of 1 N NaOH. 1.0 ml aliquots of homogenate were added to Erlenmeyer flasks equipped with a center well and a serum stopper. 0.5 ml of 1 N NaOH was present in the center well. 0.2 ml of 5 N H_2SO_4 was injected into the outer well and the CO_2 was allowed to diffuse for 3 hr. The contents of the center well were analyzed for radioactivity. Appropriate bicarbonate-^{14}C controls were run and corrections for recovery made. Each analysis was run in triplicate.

chloroform–methanol at 2 sec, but only 67% was recovered at 1 min (three rats fasted overnight per group). Since about one-fourth of the dose was unaccounted for at both 1 min and 10 min after injection of palmitate-1-^{14}C (Table 1), several experiments were performed in an attempt to account for the missing radioactivity.

At 1 min, all of the injected radioactivity could be recovered as fatty acids if rats were saponified for 1 hr at 100°C in alkaline ethanol (the mixture then being acidified and extracted with petroleum ether) instead of being homogenized in chloroform–methanol. Therefore, all of the missing ^{14}C at 1 min was in the form of lipid. Approximately 13% was recovered at 10 min as "$^{14}CO_2$" after treatment with acid and diffusion (Table 2). No experiments were done to establish (*a*) whether the $^{14}CO_2$ was free of other volatile substances and (*b*) whether substances other than bicarbonate-CO_2 had given rise to $^{14}CO_2$ under the conditions of acid diffusion at ambient temperatures. Nevertheless, these experiments indicated that radioactivity was poorly recovered at 1 min because it was in the form of a lipid which was not readily extractable with chloroform–methanol,[2] and that ^{14}C recovery was incomplete at 10 min because the palmitate had been converted to nonlipid compounds, a large portion of which behaved like bicarbonate, labile carboxyl groups, or acid-volatile substances.

Conversion of FFA-^{14}C into Hepatic TG and Non-TG Esters

The incorporation of FFA-^{14}C into hepatic lipids is given in Fig. 3. During the 2nd and 10th min of the experiment, when FFA had almost completely disappeared from plasma, ^{14}C incorporation into TG continued at a rate comparable to that found in the first 2 min of the experiment, when 93% of the FFA was being removed from the circulation. On the other hand, the other lipid esters were labeled maximally within the first 2 min of the experiment. The slow incorporation of ^{14}C into TG suggests that most of the FFA-^{14}C that was converted to TG-^{14}C first passed through a relatively large intermediate diluent pool. The pattern of isotopic incorporation into PL (and other esters) was consistent with rapid direct conversion of

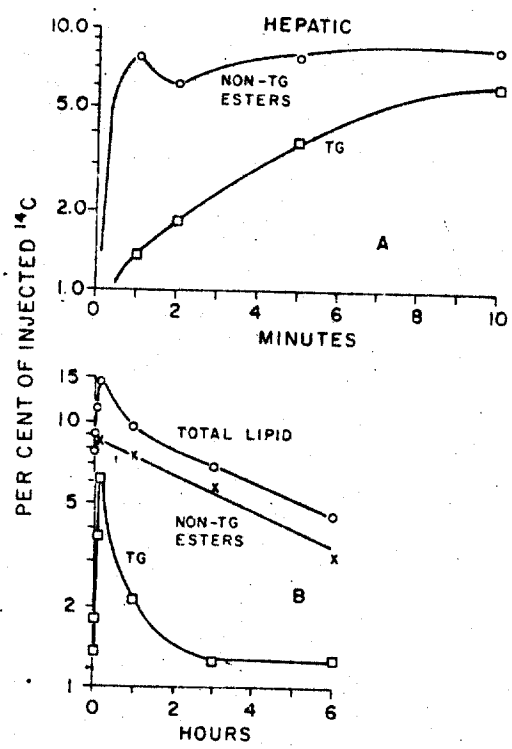


Fig. 3. Incorporation of palmitate-1-^{14}C into hepatic TG and non-TG lipid esters. Each value represents an analysis of four separate extracts, aliquots of which were pooled at each time point except at 1 min, when the individual extracts were analyzed separately. The fractional SD for ^{14}C in TG and non-TG lipids at 1 min were ±0.10 and ±0.30, respectively. Values have been corrected for trapped plasma-^{14}C. Total lipid is the sum of TG + non-TG esters. No FFA-^{14}C was recovered after corrections were made for trapped plasma. Data are plotted on a semilogarithmic scale.

FFA-^{14}C into part of this large lipid pool as an intermediate step in the formation of hepatic TG.

Maximum incorporation of ^{14}C into both TG and non-TG esters occurred within 10 min. As may be seen from Fig. 3B, over $^{2}/_{3}$ of the ^{14}C in total hepatic lipids at 10 min turned over in 6 hr. The disappearance was due largely to disappearance of TG-^{14}C in the 1st hr; however, a slow component of TG-^{14}C turnover was also observed. No significant loss of ^{14}C from hepatic TG was observed during the last 3 hr of the experiment. However, non-TG-^{14}C disappearance continued throughout the experiment at a rather constant rate ($t_{1/2}$, approximately 4.5 hr).

Conversion of ^{14}C-FFA into Extrahepatic TG and Non-TG Esters

Data similar to those shown for liver in Fig. 3 are given for extra-hepatic tissue in Fig. 4. As in liver, ^{14}C was incorporated into TG much more slowly than into non-TG lipids during the first 10 min of the experiment. The rapid component of TG turnover that was observed in liver (Fig. 3) was not found in extrahepatic tissue (Fig. 4). The curve of ^{14}C in extrahepatic non-TG esters was complex during the 1st hr; however, all values ranged between 14% and 23% of the injected dose throughout the experiment. The fractional rate constant of disappearance of ^{14}C from the TG pool during the last several hours of the experiment ($t_{1/2}$, 2.8 hr) was greater than that of either extrahepatic non-TG-^{14}C or hepatic TG-^{14}C during the same time interval.

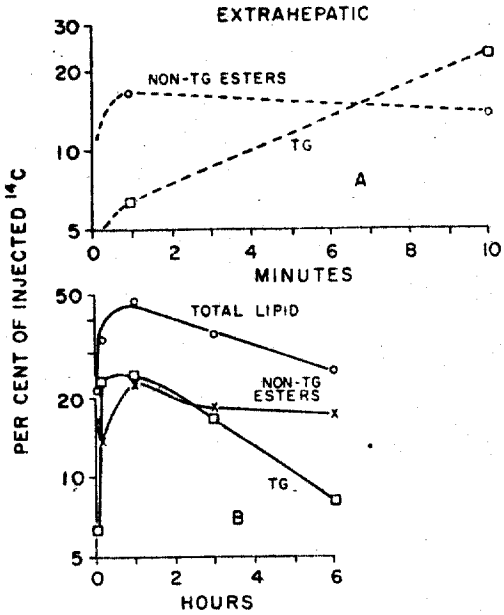


FIG. 4. Incorporation of palmitate-1-^{14}C into extrahepatic TG and non-TG lipid esters. Same numbers of rats as in Fig. 3. One value at 1 min was ignored because all radioactivity was recovered as FFA in contrast to the other samples in this and in other (unpublished) experiments. The range of values at 1 min for TG and non-TG radioactivity, uncorrected for the trapped plasma, were 7.6–10.5 and 17–31% of the injected ^{14}C. The latter range includes FFA-^{14}C which is not included as "total lipid" in the figure. As in Fig. 3, total lipid refers to the sum of TG and non-TG esters. Data are plotted on a semilogarithmic scale.

Although the total ^{14}C-lipid in extrahepatic tissue at 1 hr was five times greater than that in liver (47% compared to 9.6% of the injected ^{14}C, respectively; cf. Figs. 4 and 3), the fractional rates of disappearance were similar, $t_{1/2}$ about 4.5 hr in liver and about 6 hr in extrahepatic tissues. The fractional disappearance rate of ^{14}C from plasma FFA was similar to that of hepatic TG during t = 10–60 min and to that of extrahepatic TG during the last 5 hr of the experiment.

Turnover of Plasma Bicarbonate-^{14}C

The cumulative appearance of $^{14}CO_2$ in the breath of fasted rats after intravenous injection of bicarbonate-^{14}C is shown in Fig. 5. Practically all (95%) of the injected ^{14}C was recovered at the end of the experiment. The percentage of injected bicarbonate-^{14}C remaining in the body (100 minus values shown in Fig. 5) is plotted in Fig. 6. The curve is complex and is probably the result of several curves given by different processes including exhalation of CO_2 and mixing of extracellular bicarbonate with intracellular inorganic and organic compounds (14). The rate of CO_2 exhalation was 106 ± 21 μmoles/min per 100 g of body weight (eight rats).

Oxidation of Palmitate-1-^{14}C to CO_2

The cumulative appearance of $^{14}CO_2$ expired by fasted rats after intravenous injection of palmitate-1-^{14}C is shown in Fig. 5. Approximately $^{2}/_{3}$ of the injected ^{14}C was collected as $^{14}CO_2$ in 6 hr. The amounts of $^{14}CO_2$ expired after injection of bicarbonate-^{14}C and of palmitate-1-^{14}C during the interval 2–6 hr were 3% and 15% of the injected ^{14}C, respectively. The difference (12% of the dose) corresponds closely to the disappearance of ^{14}C from TG esters in extrahepatic tissues during the corresponding time period.

ANALYSIS AND DISCUSSION

The present study was initiated after several workers had already studied plasma FFA turnover in rats (2, 15). In each case, plasma FFA had been treated as a single homogenous pool which neither exchanged with other FFA pools nor recycled. Initial disappearance of the injected FFA-^{14}C was neither emphasized nor related to measurements of incorporation of ^{14}C into other lipids. Thus, quantitative considerations of the fate of FFA were not adequately emphasized. However, studies of FFA-^{14}C metabolism in man had indicated that FFA-^{14}C in plasma does not behave as though it were part of a single pool,

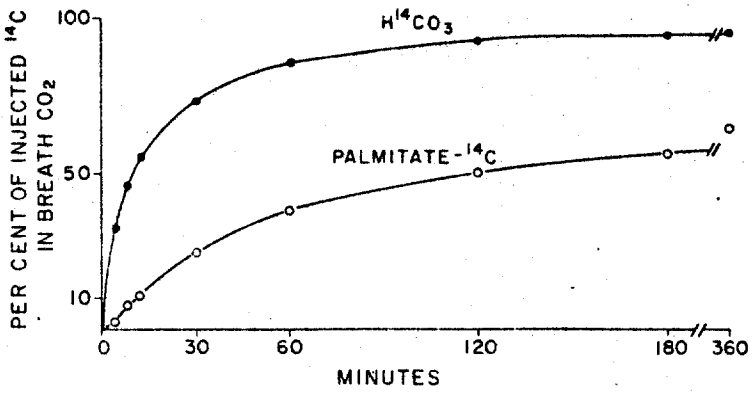


FIG. 5. Cumulative recovery of $^{14}CO_2$ in breath after intravenous injection of tracer bicarbonate-^{14}C (mean of four rats) or palmitate-1-^{14}C (mean of three rats); ^{14}C data from a fourth rat were ignored because the levels of radioactivity were so low that a technical error seemed likely. The mean fractional SD of the rates of $^{14}CO_2$ exhalation were 0.24 and 0.11 for the bicarbonate-^{14}C and palmitate-^{14}C experiments, respectively.

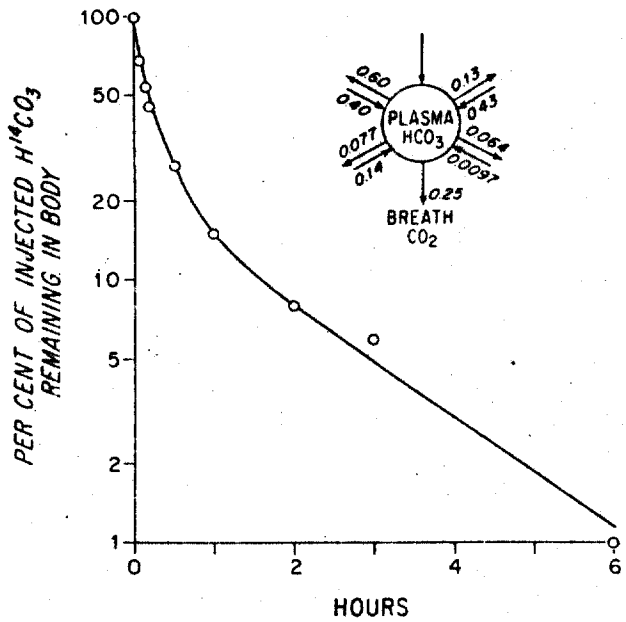


FIG. 6. Disappearance of bicarbonate-^{14}C from the body after intravenous injection of bicarbonate-^{14}C. The semilogarithmic plot of radioactivity was calculated from the data shown in the upper curve of Fig. 5 (amount remaining in body at time t = 100% − % of injected ^{14}C excreted in breath at t). These calculated values were used to derive a set of fractional rate constants which would describe operationally the kinetic behavior of bicarbonate-^{14}C as it enters the plasma. The fractional rate constants (min^{-1}) are shown in the upper right portion of the figure. The data may obviously be represented just as accurately by a simpler model; however, the subsequent analysis is independent of the model used to represent the turnover of plasma bicarbonate as long as the turnover of plasma bicarbonate is accurately described by the rate constants.

restricted to the circulation and utilized irreversibly (16). The present data show that the behavior of the FFA pool n fasted rats is also complex and that recycling of FFA is appreciable.

Several papers have appeared which attempted to account for the FFA-^{14}C which disappeared from the circulation (17–19). However, in none of these was the complex nature of the FFA disappearance curve related to the appearance of FA in esters or in respired CO_2. As a result, no integrated, thorough analysis of the quantitative significance of plasma FFA turnover in the rat has been reported. The present data are consistent with most previously published observations. The purpose of this study was to analyze our data to obtain a first approximation of rates of FFA metabolism in a common experimental animal, the rat.

All of the usual shortcomings of multicompartmental analysis apply to the present study: namely, oversimplification; reliance upon many assumptions; arbitrariness with respect to the drawing of smooth curves through scattered points and, therefore, with respect to the solution that is accepted as giving a "best fit" of the data; and compromise between enlarging the model and accepting a poor fit when two or more sets of data are not exactly compatible with a relatively simple model. Although our aim has been to gain some understanding of quantitative aspects of lipid metabolism in the rat, our calculations are only intended to be estimates which apply to rats used under the specific experimental conditions that we employed. These first approximations are a basis for further work, and not absolute values. Analysis and discussion of the observed data have been arranged as follows:

List of assumptions used in calculations
Model of FFA metabolism in fasted rats
Multicompartmental analysis (fractional rate constants of flux)
Specific estimates of selected rates
Discussion of the physiological and biochemical significance of estimated rates as they are presented

Assumptions

The multicompartmental analysis is based upon the following assumptions. Some brief comments have been added parenthetically after each assumption. Compartment numbers are defined in Fig. 10.

1. FFA in compartments *1* and *2* and total body bicarbonate pools did not change in concentration. Similarly, hepatic TG and non-TG pools were in a steady state. [No large changes in plasma FFA concentration were noted during a separate, 10 min study of FFA metabolism.[3] We have never observed increases such as the 240% change reported by Carlson and Nye (20) 15 min after a subcutaneous injection of saline.]

2. Esterification and oxidation of FFA stopped as soon as animals were placed in liquid nitrogen. [Since complete freezing of a rat carcass in liquid nitrogen is known not to be instantaneous, observed values of ^{14}C incorporation into extrahepatic lipid esters will tend to be erroneously high.]

3. No FFA need enter compartments *1*, *2*, or *3* during oxidation of lipid esters to CO_2. [Enzymes that can hydrolyze lipid esters and can activate and oxidize fatty acids are located intracellularly; it seems plausible that fatty acids formed from esters can be oxidized to CO_2 without first having to equilibrate with relatively large pools of FFA.]

4. All net input of FFA enters compartment *1* (Fig. 11) without first mixing with extraplasma FFA; newly formed FFA is not appreciably labeled during the experiment. [Stein and Stein have shown that FFA that is converted to TG by adipose tissue is diluted to such an extent that little radioactivity leaves the tissue during brief periods of fasting (21, 22). However, there is still little information regarding the extent to which FFA newly formed from TG mixes with intracellular FFA pools in adipose tissue.]

5. ^{14}C that was not recovered at 1 min in chloroform–methanol (Table 1) was in the form of extraplasmal, bound, but exchangeable, FFA (compartments *2* and *3*; Fig. 11). [The plasma FFA-^{14}C curve indicates that rapid exchange of plasma FFA with some other pool of lipid occurs. One possibility is that plasma FFA exchanges rapidly with extraplasma FFA as discussed below in Specific Estimates of Selected Rates (*b*). More FFA-^{14}C was recovered from whole rats at 1 min than was present in plasma. Therefore significant quantities of ^{14}C must have been present in an unesterified form in tissues at 1 min. The missing ^{14}C was in the form of lipid and could be extracted if animals were homogenized in alkaline ethanol (Table 2). No difficulty was encountered in extracting esterified lipids between t = 1 and 6 hr when FFA-^{14}C had virtually disappeared. Moreover, recovery of FFA-^{14}C was quantitative at 2 sec (Table 1) when almost all the labeled FA was probably still in the plasma. We infer from this that the labeled FA was in a unique form at 1 min which made it difficult to extract. Unpublished experiments have indicated that occasionally quantitative recoveries of ^{14}C could be achieved. When this occurred, the "extra" radioactivity was associated with the FFA fraction.[2]]

6. The slowly exchanging component of the plasma FFA-^{14}C curve reflects primarily the slow exchange of plasma FFA with extrahepatic tissue TG by a process which involves no net formation of FFA. [We have obtained no information on recycling other than that it occurs and that it could represent a very rapid process relative to the turnover rate of plasma FFA. All estimates of irreversible disposal, oxidation, and esterification rates must be reduced if recycling of FFA-^{14}C is associated with a net efflux of FFA from nonadipose extrahepatic tissues. Hepatic lipids were assumed not to recycle FFA-^{14}C into plasma; however, this could occur (23).]

7. CO_2 formed during the oxidation of FFA diffuses directly into blood and is expired in the same manner as bicarbonate-^{14}C which is injected directly into plasma. [The basis of this assumption is that CO_2 which forms intracellularly diffuses into red blood cells and into plasma before it mixes with intracellular pools of bicarbonate (14).]

8. All intermediates in the esterification and oxidation of fatty acids that are not shown in Fig. 10 are so small and rapidly turning over that they may be ignored mathematically. [Pool sizes of acyl CoA and of the tricarboxylic acid cycle intermediates are known to be small enough to be consistent with this assumption. However, it is not known to what extent intermediates in the tricarboxylic acid cycle exchange their carbon with large pools of amino acids in the cells that are oxidizing fatty acids to CO_2. Evidence has been presented in humans that acetate is oxidized to CO_2 by way of very rapidly turning over pools (24).]

9. The model itself represents correctly the relationships among FFA, TG, non-TG esters, and CO_2 in the fasted rat. [This assumption is wrong, because of the deletion of additional liver TG pools and of plasma TG and non-TG pools. The model is obviously oversimplified and erroneously treats a highly complex system in a simple manner. The relationships between TG and non-TG esters are not known and the data obtained here do not establish whether or not the model we have chosen is valid.]

10. Palmitate-1-^{14}C added to serum as a potassium salt in aqueous ethanol is complexed to serum albumin like other plasma fatty acids; the fractional rate constant

[3] N. Baker and H. Rostami. Manuscript in preparation. The formula for irreversible disposal rate (R) is: $R = Q_1/\Sigma(H_i/g_i)$, where Q_1 = pool size of plasma FFA, and H_i and g_i = the intercepts and slopes of the plasma FFA-^{14}C–time curve, respectively.

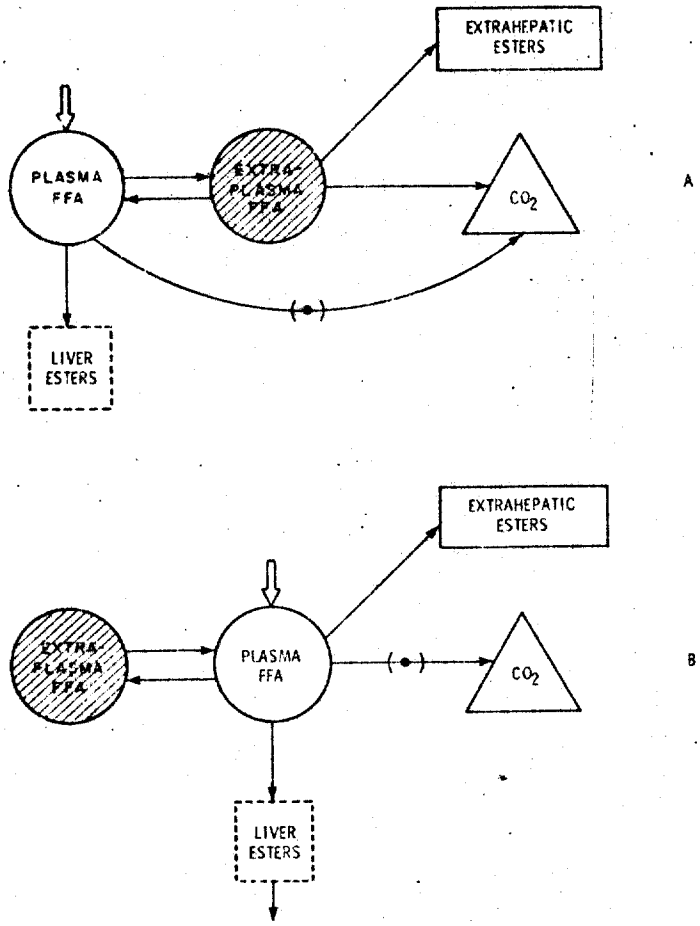


FIG. 7. Two simple models which describe possible relationships of extraplasma FFA and to other products of FFA metabolism. See text.

of turnover of the tracer and the metabolic pathways followed by the labeled fatty acid reflect the behavior of all plasma fatty acids. [We have no information to support this assumption other than the observation that labeled fatty acids in the serum obtained 5 min after injection of the artificial complex (Fig. 2) disappeared from plasma at about the same fractional rate as the initial complex (Fig. 1). The reinjected material was probably labeled in a physiological manner. However, evidence has been presented recently that the rate of palmitate-1-^{14}C disappearance from the circulation of dogs varies according to the method used to complex the tracer with albumin (25).]

11. TG are formed from fatty acids in the same cells as are non-TG esters. [This could be true for native fatty acid albumin and not for an artificial complex. See assumption (10).]

Model of FFA Metabolism

Minimal models of FFA metabolism in fasted rats as defined by other workers are shown in Figs. 7 A and B. Both models A and B take into account experimental evidence that shows that plasma FFA is converted to CO_2 (26, 27) and to lipid esters (2, 19) in both hepatic and extrahepatic tissues. Some evidence suggests that oxidation of FFA to CO_2 occurs primarily in extrahepatic tissues (17, 26); however, conversion of FFA within the liver to intermediate compounds such as ketone bodies which are subsequently oxidized to CO_2 in extrahepatic tissues is also known to occur (27) and is indicated by the symbol (—·→). Plasma FFA is shown to be exchanging with extraplasma FFA. In Fig. 7A, but not in 7B, the exchangeable FFA pool outside of plasma is considered to act as a diluent through which FFA derived from plasma must pass before it is metabolized by extrahepatic tissues. As Olivecrona pointed out, both types (Fig. 7A and 7B) of exchange probably occur. A large, slowly exchanging pool may exist in adipose tissue (15); a small, rapidly exchanging FFA compartment exists in interstitial fluid and, perhaps, also in the capillary endothelium and extrahepatic cells (28, 29). This is depicted in the simple model shown in Fig. 8. An important feature of this model is the net inflow of FFA, which is shown entering the plasma FFA compartment without first undergoing dilu-

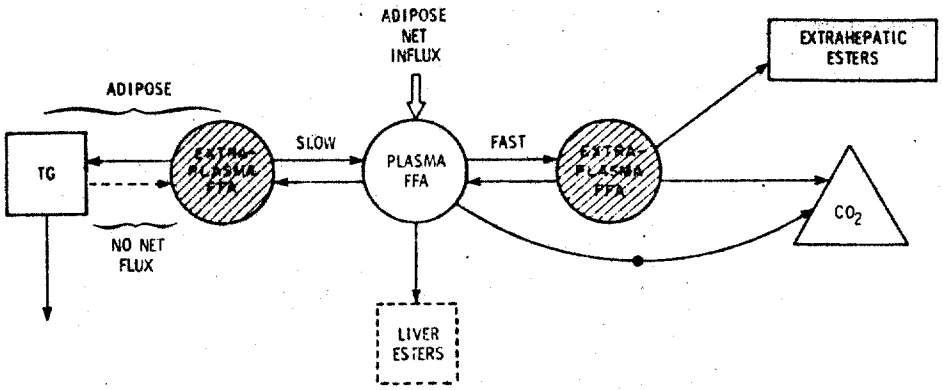


FIG. 8. Simplified model of FFA metabolism. The figure represents a composite of the two models shown in Fig. 7. Two arrows depicting the movement of FA from adipose tissue into plasma FFA are shown. One of these, to the left, represents a hypothetical exchange or mixing phenomenon with no net flow (see assumptions). The broken line from adipose TG to adipose FFA is also assumed to represent an exchange process. The net influx from adipose TG to plasma FFA is assumed to contain negligible radioactivity as depicted by the arrow at the top of the circle representing the plasma FFA compartment. The interchange of plasma with two extraplasma FFA compartments is shown. The exchange was assumed to be relatively fast in nonadipose tissue and slower in adipose tissue.

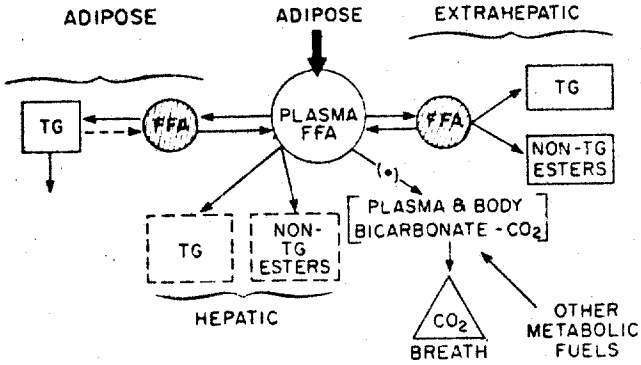


FIG. 9. Expansion of model shown in Fig. 8 to illustrate the final model which served as a basis for the experimental design. Note the simple branching of plasma or extraplasma FFA into TG and non-TG esters in hepatic and extrahepatic (nonadipose) tissues, respectively. The plasma and body bicarbonate pools were shown in Fig. 6 and have been deleted here for simplicity. Also, the exits from hepatic and extrahepatic lipids indicating turnover of these lipids are not shown here but were considered in the subsequent analysis.

tion in the relatively large adipose tissue FFA pool (see assumption 4, above). Some exchange of ^{14}C is considered possible between adipose TG and plasma FFA via the intracellular adipose tissue FFA pool (15) as indicated in Fig. 8.

In order to take into consideration the conversion of plasma FFA to its major initial products, TG and PL, in hepatic and extrahepatic tissues we expanded the model of Fig. 8 to that of Fig. 9. Although FFA must pass through intermediate pools before being incorporated into fatty acyl esters, these pools are not shown because they are thought to be turning over so rapidly that they may be considered negligible from a mathematical standpoint. Any CO_2 formed in the body has been assumed to enter plasma (14, 30) and to be excreted into breath in the same manner as plasma bicarbonate. A five pool model, not shown, was employed to represent the plasma and body bicarbonate system and the mathematical function was defined experimentally in a separate experiment (Fig. 6).

The model of Fig. 9 was found to be incompatible with the data. Most of the palmitate-^{14}C was apparently incorporated into a relatively large, slowly turning over, non-TG ester pool as an intermediate step during formation of TG in both hepatic and extrahepatic tissues (cf assumption 11 above). It was then necessary to employ a model such as that shown in Fig. 10 in order to find a set of rate constants that would describe the flow of plasma FFA to and from hepatic and extrahepatic lipids and to CO_2. According to the model of Fig. 10, FFA may enter TG by two possible pathways: (*a*) direct esterification via rapidly turning over intermediates (not shown); (*b*) indirect esterification, via compartments 5 and 9, which exchange FA with both TG and non-TG esters, but which convert incoming FA to non-TG esters much more rapidly than to TG esters. This model provides for a mechanism that would account for the observed rapid incorporation of palmitate-^{14}C into non-TG esters followed by a relatively slow incorporation of ^{14}C into TG esters. In addition, the possibility of recycling of extrahepatic TGFA into plasma FFA has been included in the model of Fig. 10, which served as the basis of subsequent calculations.

Multicompartmental Analysis of the Data (Fractional Rate Constants of Flux)

The data of Figs. 1–6 and Tables 1 and 2 are in excess of the minimum required to calculate, uniquely, a set of

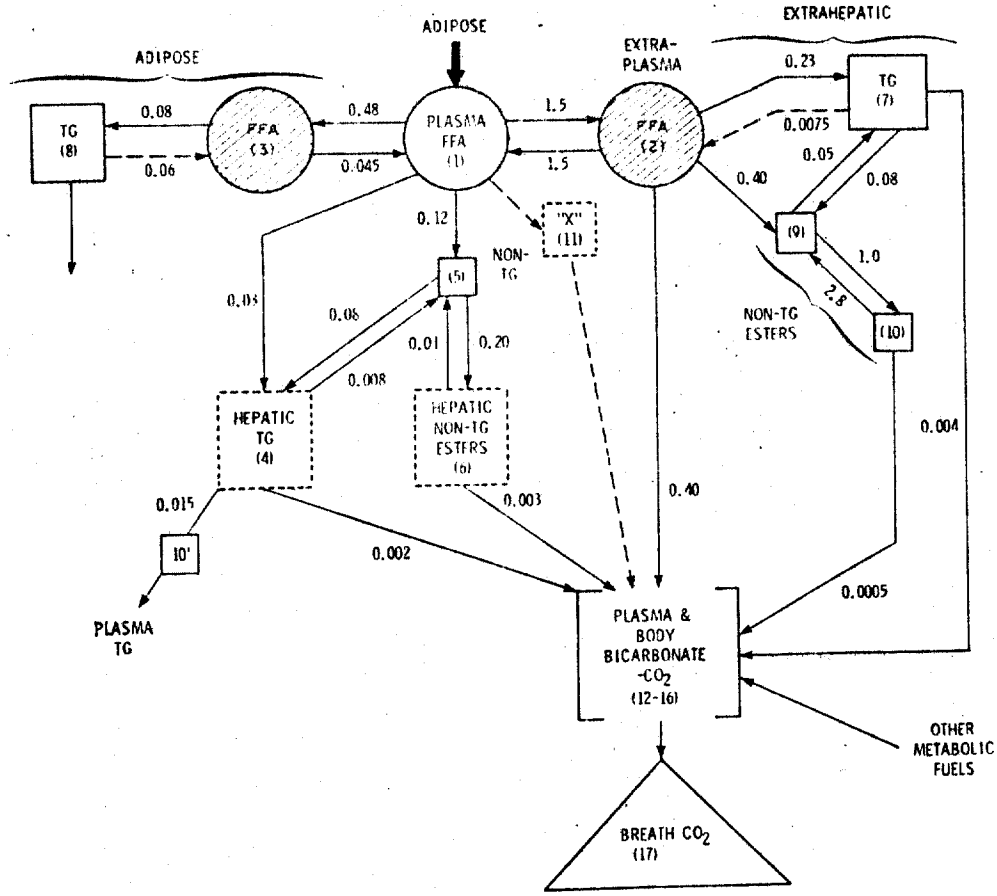
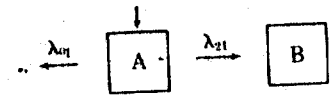


FIG. 10. Final model used to analyze the data. Fractional rate constants (min^{-1}) which were derived by the program of Berman et al. (33) are shown for all arrows except the broken arrows into and out of compartment *11* (see text). However, not all fractional rate constants were uniquely defined by the data even if the model is assumed to be correct. In particular, the interflow among compartments *4*, *5*, and *6* and among *7*, *9*, and *10* could not be established by the present data. Some of these compartments and arrows were required to depict the unexpected, slow accumulation of ^{14}C into TG as compared to non-TG lipid esters. A broken arrow is also shown from extrahepatic TG (compartment *7*) to extraplasma FFA (compartment *2*). This arrow was inserted to indicate a slow recycling of FA from TG. Although the present data demonstrate clearly that recycling of plasma FFA occurs, neither our experimental results nor those published by others establish the compartments (hepatic or extrahepatic, adipose or nonadipose, TG or non-TG) which are involved in the recycling phenomenon. Havel and Goldfien showed that some recycling occurs both in hepatectomized and in eviscerated dogs (31). It is also not clear from the data whether or not recycling of FFA-^{14}C is associated with a net influx of FFA into plasma.

fractional rate constants of FFA exchange, esterification, and oxidation using a model such as that shown in Fig. 7. Theoretically (32), the only measurements required would be radioactivity against time in plasma FFA (Fig. 1) and one early measurement of radioactivity in liver esters and in exhaled CO_2. Instead, data have been obtained at five times for liver esters and at four times for CO_2. Moreover, there are data available at five times for extrahepatic esters.

Successive independent calculation of all the fractional rate constants shown in Fig. 10 could be achieved, theoretically, using the data of Fig. 1 plus two data points, then with two others, and so on until separate independent values were obtained for each rate constant. An average value and standard deviation of each rate constant could then be calculated. The digital computer program of Berman, Weiss, and Shahn (33), which we have used, gives an equivalent average value and standard deviations for each rate constant by treating all of the data simultaneously. The reader may see this more readily if he considers the following simple, two pool model



in which B turns over either (*a*) very slowly relative to A, (*b*) not at all (as in the case of an end-product), or (*c*) at a fractional rate which is rapid, but known. In each case, measurement of radioactivity in *A* [$A^*(t)$], after injecting tracer [$A^*(0)$] at zero time into *A*, plus one measurement of radioactivity at any time in *B* [$B^*(t)$] is sufficient to allow estimation of λ_{01} and λ_{21}. If, instead, a complete curve (many values), which defines $B^*(t)$, is obtained, then each extra value of $B^*(t)$ together with the data of

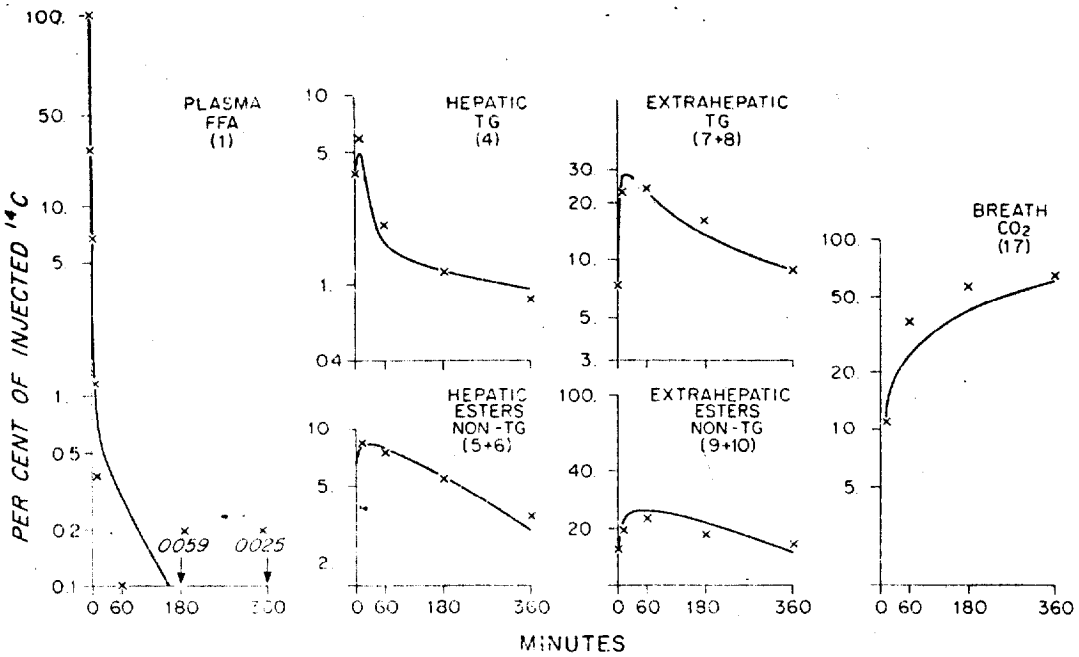
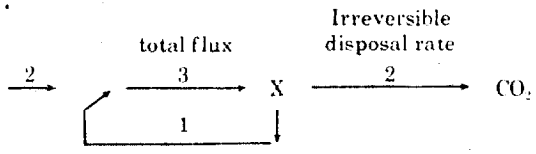


FIG. 11. Comparison of experimental data with ^{14}C time curves derived by the digital computer on the basis of the model and fractional rate constants shown in Fig. 10. The solid lines represent the calculated curves obtained by the program of Berman et al. (33). Experimental data are indicated by *x*. The data for extraplasma FFA were not included in the analysis because of the difficulties encountered in extracting the FFA at the early times, especially at 1 min.

$A^*(t)$ may be used to calculate, independently, both the fractional rate constants and standard deviations for each. Similarly, when a model such as that shown in Fig. 9 is used, rates of TG and of non-TG ester formation may be calculated uniquely given only a single early value of hepatic and extrahepatic TG and non-TG esters. Special care was taken to insure that data were in excess of the minimal requirements in order to obtain a valid estimate of these rates. However, as noted above, the data were incompatible with this model. Using the more complex model of Fig. 10 we obtained an acceptable solution, which is also given in Fig. 10. The data do not define uniquely the fractional rate constants of movement of fatty acids among the several hepatic and extrahepatic lipid ester compartments. Some of these values (see legend to Fig. 10) are presented only so that the reader may check the analysis and as a basis for further investigation. The quality of fit is illustrated in Fig. 11 in which some of the observed data are compared with values calculated by a digital computer (33) using the fractional rate constants shown in Fig. 10. Although there are some minor discrepancies between observed and calculated values, no effort has been made to make additional fine adjustments of the estimated rate constants and of the model, since these either would have little effect upon the final values or else the adjustments would be arbitrary and meaningless without additional data. One compartment, which we have not considered in our calculations, but which should probably be taken into account, is compartment *11* (Fig. 10). The data of Table 2 and for exhaled CO_2 together with published experiments all indicate that this route of FFA metabolism, which would include oxidation of FFA to CO_2 via ketone bodies formed in the liver, should be included in the model.

Specific Estimates of Selected Rates

(*a*) *Irreversible Disposal (Turnover) Rate of FFA.* The concept of irreversible disposal rate was first introduced in order to distinguish between the net rate of formation (or utilization) of a substance and the total flux of the substance (34). This is illustrated in the following example, which shows substance *x* having a total flux of 3 μmoles/min, a recycling rate of 1 μmole/min, and an irreversible disposal rate of 2 μmoles/min. The irreversible disposal



rate in this case is only $^2/_3$ of the total flux. In a constant infusion experiment, which usually does not take recycling into account, the turnover rate corresponds to the irreversible disposal rate of a single injection experiment.

The irreversible disposal rate of FFA was estimated to be 2.9 μmoles of FFA/min per 100 g body weight, from

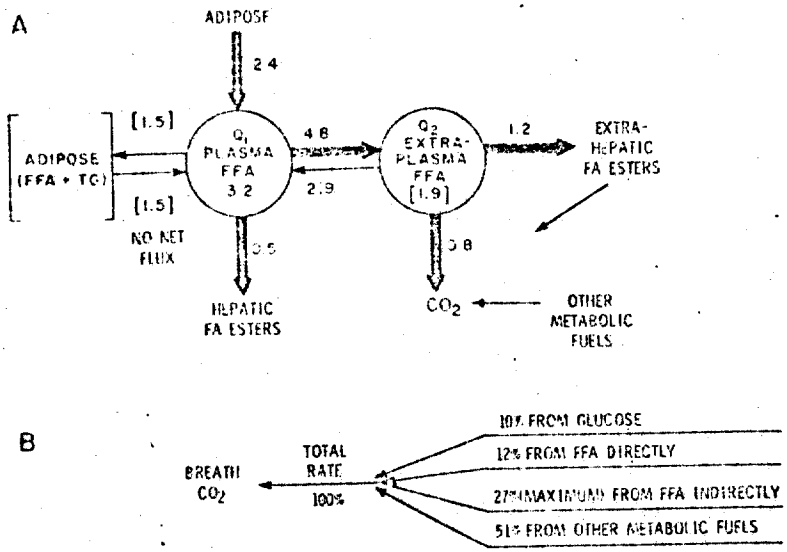


FIG. 12. Rates of plasma FFA exchange, esterification, and oxidation. The complex model of Fig. 10 was simplified to the model shown in A, above, and the estimated rates of flux were calculated on the basis of the appropriate fractional rate constants (Fig. 11) and the plasma FFA pool size. Rates are expressed as μmoles of fatty acid/min per 100 g body weight. Pool sizes are given as μmoles of fatty acid/100 g body weight. Pool size Q_2 is shown in brackets to indicate that the calculated value is a minimum estimate (see text). The lower part of the figure, B, relates the estimated rates of FFA oxidation by direct and indirect pathways to the total rate of CO_2 oxidation.

the data of Fig. 1 and the measured plasma FFA pool size.[3] It should be noted that the calculation does not require construction of a model provided that all newly formed FFA with which plasma FFA mixes enters the plasma before mixing with other FFA pools. As will be seen later, a complex multicompartmental analysis of the data resulted in a somewhat lower estimate (2.4 μmoles/min per 100 g body wt).

(*b*) *Rates of Plasma FFA Exchange with Extraplasma FA, and Net Rate of FFA Mobilization Based upon a Multicompartmental Analysis.* In Fig. 12 a summary of estimated rates of FFA exchange and net turnover is presented. In the absence of a complete balance of activity at early times, there was about ±25% uncertainty with respect to estimated rates of exchange and extraplasma FFA pool size.[4] However, it is clear from the experimental data that a major fraction of the FFA that left the plasma in the 1st min did not appear as easily extractable FA ester, but rather recycled into the circulation almost as fast as it had left. The uncertainty would be far less if all the activity recovered at 1 min (Table 2) and not accounted for in plasma FFA or in hepatic and extrahepatic esters were known to be in the form of extraplasma FFA (as we have assumed). Nevertheless, the total disposal of fatty acid is probably depicted accurately (±25%)[4] by the values (per 100 g body wt) of 1.2 μmoles of FFA esterified per min by extrahepatic tissues, 0.8 μmole of FFA oxidized to CO_2 per min, and 0.5 μmole of FFA esterified in liver per min. The total rate of net FFA mobilization (irreversible disposal) was 2.4 μmoles/min per 100 g body wt. If another 0.5 μmole/min were converted by liver to water-soluble compounds such as glucose and ketone bodies which were subsequently oxidized to CO_2, the irreversible disposal rate would agree with the value calculated from the plasma FFA curve alone (see "Irreversible Disposal Rate of FFA," above) and would improve the correspondence between observed and calculated values for $^{14}CO_2$ shown in Fig. 11.

The size of the most rapidly exchanging extraplasma FFA pool (Q_2, Fig. 12A) was estimated to be slightly more than half that of the plasma FFA pool (Q_1). This is consistent with reports that both albumin and fatty acids exist in interstitial fluid and at a lower concentration than in plasma (28, 29). The calculation of Q_2 was based upon steady state considerations, simplification of a portion of the model in Fig. 10 to that shown in Fig. 12A, the fractional rate constants given in Fig. 10, and the measured plasma FFA pool size: $Q_2 = Q_1\lambda_{21}/\lambda_{22}$, where λ_{21} = fraction per min into compartment *2* from *1*, and λ_{22} = fraction per min out of compartment *2* by all pathways. If our assumption is incorrect, and if a net inflow, I_2, from extrahepatic FA esters into Q_2 were known and taken into account, a larger estimate of Q_2 would be obtained: $Q_2 = (Q_1\lambda_{21} + I_2)/\lambda_{22}$. In Fig. 12A the plasma

[4] This is a crude estimate of error based upon computer analyses in which fractional rate constants which determine the early disappearance and return of FFA-^{14}C from compartment *1* were systematically varied and the resulting curve for compartment *1* compared with the experimental data. When these rate constants were increased or decreased by 25%, the resulting curves were clearly incompatible with our data.

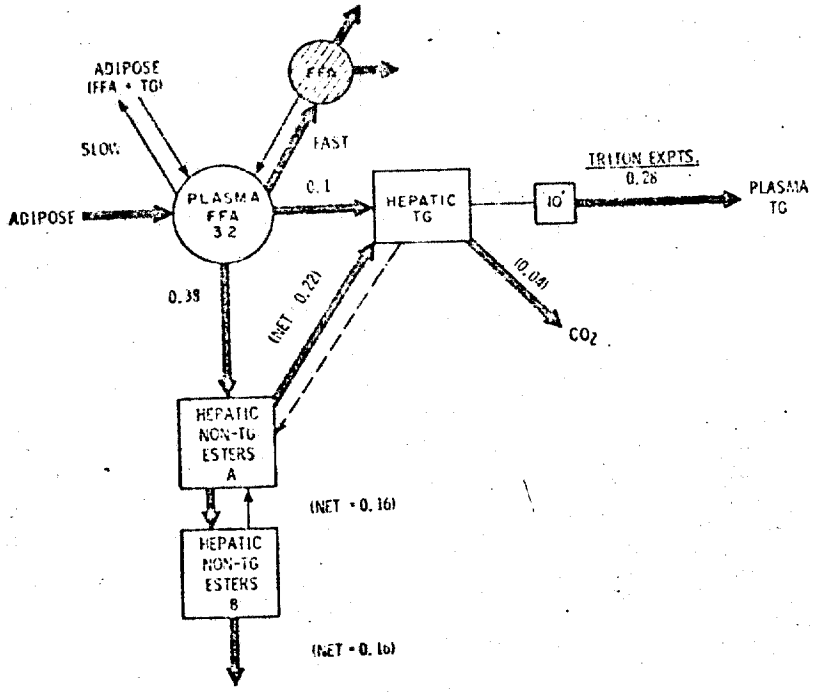


FIG. 13. Rates of hepatic TG formation by direct and relatively indirect pathways. The rates and pool sizes are expressed in the units given in Fig. 12. The rate of TG secretion is taken from separate experiments (see text) and is a minimum estimate. The rate of direct hepatic TG formation from plasma FFA was found to be only a fraction of the total rate of TG formation. The remainder of the required TGFA (0.22 μmoles/min of TGFA per 100 g body wt) was assumed to come from non-TG esters which were formed from plasma FFA at a rate sufficient to supply the required net flow of FA.

FFA is shown to exchange relatively slowly with a large pool of extraplasma FFA and TG, which we have assumed to be in adipose tissue. The exchange rates are highly uncertain (values in brackets). We emphasize that, although FFA being produced by adipose tissue is shown to be undiluted by the large adipose tissue FFA pool before it enters the circulation, we have presented no experimental data to support this assumption. In the absence of other data, this treatment simplifies calculations.

(*c*) *Oxidation of FFA to CO_2.* The value of direct FFA oxidation to CO_2, 0.8 μmole/min per 100 g body wt (Fig. 12), represents approximately 33% of the total irreversible disposal of plasma FFA. It also accounts for approximately 13 μmoles of CO_2/min per 100 g body wt (assuming 17 atoms C per molecule of FFA, average) which amounts to only 12% of the total CO_2 actually formed each minute. If the maximal FFA irreversible disposal rate (based upon the FFA-^{14}C curve and plasma FFA concentration[3]) is considered to represent the rate of oxidation of fatty acids to CO_2 by all direct and indirect pathways (including FFA → hepatic PL → hepatic TG → plasma TG → extrahepatic lipid → CO_2), then approximately 39% of the observed CO_2 expiration may be accounted for. Since only about 10% of the CO_2 formed by fasted rats is derived from glucose by relatively direct oxidative pathways (35), about 50% of exhaled CO_2 must be derived from other metabolic fuels such as protein, amino acids, and lipids which were being oxidized faster than they were replaced. These relationships are summarized in Fig. 12 B.

All estimates of rates of oxidation are dependent upon the assumption that CO_2 formed intracellularly enters the plasma bicarbonate immediately and that the subsequent mixing and exhalation kinetics are identical with those exhibited by intravenously injected bicarbonate (14, 30). This assumption has never been adequately tested; furthermore, the amount of bicarbonate recovered in the whole animal at 10 min (13% of the injected dose, Table 2) was an order of magnitude greater than would be predicted if the model and rate constants shown in Fig. 10 were used. Further comparisons of predicted and measured total body bicarbonate-^{14}C after intravenous injection of bicarbonate-^{14}C and of other labeled metabolic fuels should prove enlightening.

(*d*) *Rates of Hepatic TG Formation from Plasma FFA by Direct and Indirect Pathways.* The maximum incorporation of FFA-^{14}C into hepatic TG was less than half the values previously reported (1). This observation was the basis of a subsequent study, which showed that the incorporation of FFA-^{14}C into liver TG was markedly stimulated by ether anesthesia (36). However, the most striking observation in the present study was the slow rate of for-

mation of hepatic and extrahepatic TG relative to the rapid disappearance of FFA-^{14}C and to the rapid appearance of radioactivity in other esters, mainly PL.

From the present data the net rates of TG and non-TG ester synthesis from plasma FFA by direct pathways could be estimated. Furthermore, by including data from a separate study of TG secretion in unanesthetized, fasted rats[5] we could get approximate estimates for the quantitative relationships between hepatic TG synthesis and secretion. These calculations are summarized in Fig. 13. The poorly defined fractional rate constants of interchange between compartments *4*, *5*, and *6* in Fig. 10 were not necessary for the calculation of these rates. More than enough FFA was taken up by the liver each minute to account for a net TG secretory rate of 0.28 μmole of TGFA per min per 100 g body wt, which was the average value found in a recent study of post-Triton hypertriglyceridemia in fasted rats.[5] However, only 36% of the TGFA secreted was synthesized directly from plasma FFA. The remaining 64% apparently was formed by a route that entailed prior synthesis of a non-TG ester. The calculated percentage of newly synthesized hepatic TGFA formed directly from plasma FFA may be taken as a maximum estimate since this value is based upon an assumed rate of hepatic TG secretion that is considerably below values which have been reported in the literature. This lends support to an earlier suggestion (1) that a major fraction of the net TG synthesized in liver may be derived from relatively large pools of plasma fatty acid, which are replaced by net synthesis from plasma FFA in fasted rats. However, the reader should bear in mind that all calculations are dependent upon many assumptions.

(*e*) *Conversion of Plasma FFA to Extrahepatic TG and Non-TG Ester.* Our experimental design was based upon the hypothesis that FFA would enter extrahepatic TG and non-TG by a branching pathway such as that shown in Fig. 14A. The branching point was assumed to be a small, rapidly turning over pool (Fig. 14A, ●) that could be ignored mathematically, with consequent reduction of Model A to Model B (Fig. 14). The observed data were not compatible with Model B. A more consistent model is shown in Fig. 14C. Even this is a simplification of the more complex model which gave the best fit (Fig. 10). Without an independent measurement of the net rate of extrahepatic TG turnover rate (μmoles min of TGFA per 100 g of body wt) it was not possible to calculate the net rate of TG formation by indirect pathways. If it is assumed that no net FA formation occurs from extrahepatic TG, one may calculate from the values shown in Figs. 11 and 12 that a minimum of 1.2 μmoles of FFA per min were esterified in extrahepatic tissue per 100 g body

[5] N. Baker, A. S. Garfinkel, and M. C. Schotz. Manuscript submitted for publication. The rate of triglyceride secretion shown in Fig. 13 is taken from this study.

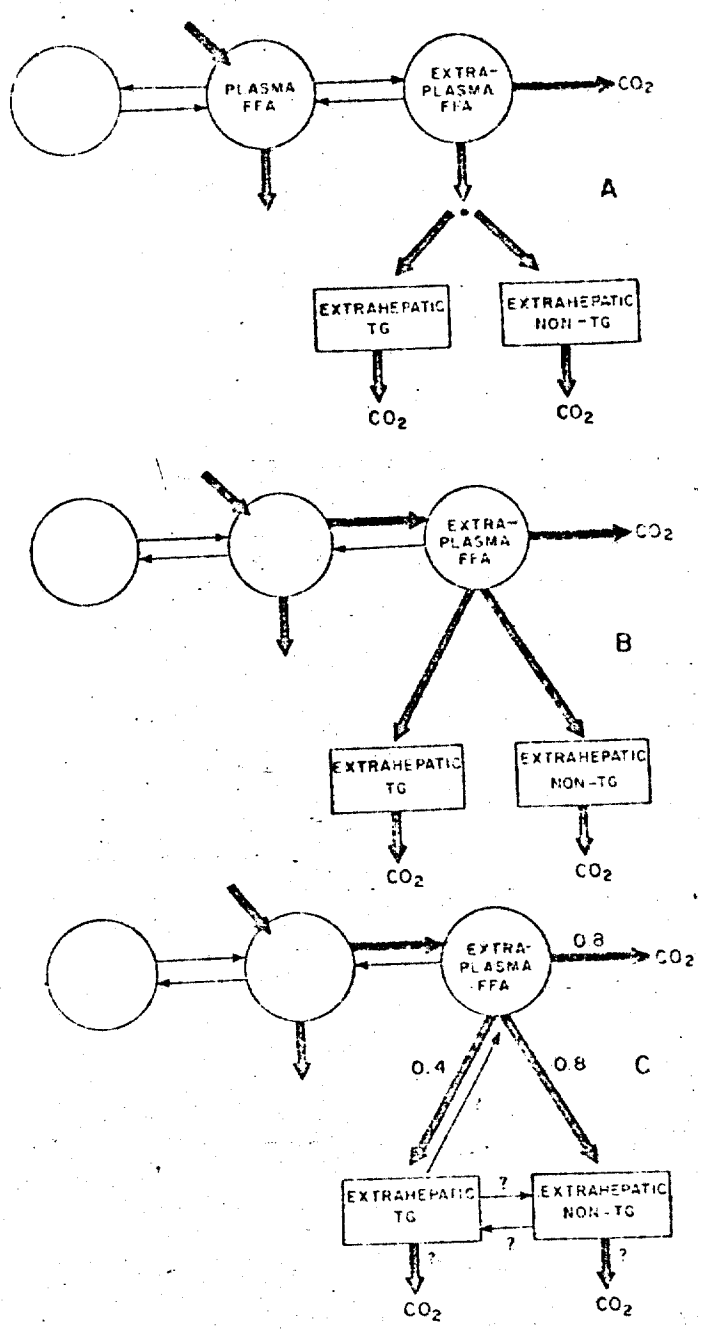


FIG. 14. Estimation of rates of TG and non-TG formation from fatty acids in extrahepatic tissues. A shows that part of the initial model which was to have served as a basis for calculating net rates of esterification after simplification to model B (see text). However, the data indicated that a more complex model such as that shown in C may be required to describe the interrelations between extraplasma FFA and extrahepatic TG and non-TG esters. The data obtained could not define rates of flow shown by question marks. However, if it is assumed that no net extraplasma FFA is formed from TG or non-TG esters in nonadipose, extrahepatic tissues, then the rates of direct esterification shown in C may be calculated from the fractional rate constants shown in Fig. 11 and the pool sizes shown in Fig. 12. Units are as given in Fig. 12.

weight. One-third entered TG directly and the other two-thirds was incorporated into other lipids. Extensive exchange of FFA-^{14}C appeared to take place between TG and non-TG lipids. It was not possible to estimate the actual rates of exchange, oxidation to CO_2, and recycling to FFA in terms of μmoles of esterified FA per min per 100 g body weight. If we assume that no net FA was deposited as lipid esters in extrahepatic tissues of fasted rats, then at least 1.2 μmoles of FA ester per min per 100 g body wt were either recycled to FFA or were oxidized to CO_2 or both. The maximum fractional rate of oxidation to CO_2, defined by the measured CO_2 curve and the decay of the extrahepatic lipid-^{14}C curves, could not have been more than 0.4% and 0.05% per min in the case of TG and non-TG, respectively. If the 0.4 and 0.8 μmole FA per min per 100 g which entered TG and PL, respectively, were not recycled but rather were oxidized to CO_2, the pool sizes of TG and PL would be approximately 100 μmoles of (TG)FA per 100 g body wt and 1600 μmoles of non-TG esterified FA per 100 g body wt. These estimates assume no net flow of FA between TG and non-TG. Further work in the direction of defining rates of FA recycling, exchange, and metabolism in extrahepatic tissues will require more refined methods of sampling and isolation of interstitial fluid FFA, and of halting postmortem esterification during early periods of the experiment. Individual tissues should be sampled and further knowledge of the actual compounds and mechanisms of (TG)FA and of (non-TG)FA must be understood so that the appropriate metabolites may be isolated and assayed.

The present analysis is a limited and very elementary attempt towards ultimately defining the rates of FFA oxidation and esterification under various physiological conditions. We have not considered important points such as ketone body formation and gluconeogenesis from fatty acids in liver. However, the model has already proved useful as a framework for further work in our own laboratory and is presented in the hope that it will stimulate others to formulate new hypotheses and to design future experiments that will yield information regarding quantitative aspects of in vivo fat metabolism.

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Route of Absorption and Distribution of Oleic Acid and Triolein in the Rat

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During the last few years a better understanding of the process of intestinal fat absorption has been gained, largely with the aid of isotopically labelled material, in combination with the technique of Bollman, Cain & Grindlay (1948) for collecting intestinal or thoracic lymph from unanaesthetized rats. The results of these investigations have shown that after absorption long-chain saturated fatty acids are mainly transported via the intestinal lymph as triglycerides both in rats and cats (Bloom, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Bergström, Borgström, Carlsten & Rottenberg, 1950; Bloom, Chaikoff, Reinhardt & Dauben, 1951; Chaikoff, Bloom, Stevens, Reinhardt & Dauben, 1951; Bloom, Chaikoff & Reinhardt, 1951; Borgström, 1951, 1952*a*, *b*, *c*; Bergström, Borgström & Carlsten, 1954). Fatty acids with 10 or less carbon atoms are, however, mainly transported from the intestine via the portal blood vessels (Bloom, Chaikoff & Reinhardt, 1951; Kiyasu, Bloom & Chaikoff, 1952) largely in free form (Borgström, to be published). For summaries see Bergström & Borgström (1953, 1954).

The higher saturated fatty acids are thus not absorbed via different routes according to the state in which they enter the intestinal mucosa, i.e. as free acids or esterified with glycerol, as postulated in the original fat–fatty acid partition theory of Frazer (Frazer, 1938, 1943, 1946, 1948*a*, *b*, 1950, 1951, 1952).

There is also much experimental evidence that the unsaturated long-chain fatty acids are mainly absorbed via the lymphatics irrespective of whether they are fed as free acids or glycerides.

Munk (1880) fed oleic acid to dogs with a cannulated thoracic duct and found that there was a considerable increase in the neutral fat of the lymph. Munk & Rosenstein (1891) fed olive oil, oleic and erucic acid to a patient having a lymphatic fistula and obtained a high recovery of the ingested fat in the lymph. Freeman & Ivy (1935) obtained the same results after feeding olive oil to dogs. Bollman, Flock, Cain & Grindlay (1950) observed a marked increase of glycerides in the lymph after feeding oleic acid to dogs. Reiser & Bryson (1951) and Reiser, Bryson, Carr & Kuiken (1952) fed linoleic acid with conjugated double bonds to rats having a thoracic duct fistula and recovered part of the labelled acid in the lymph fat both after free acid and glyceride ingestion.

On the other hand, Frazer (1943), after feeding olive oil and oleic acid to rats and assessing the results with a technique mainly based on chylomicron counts and histological observations, concluded that 'fatty acid passes by the portal vein to the liver while neutral fat goes by the lymphatic route...' and this opinion has been reiterated in many subsequent publications (see above). Tidwell (1950), however, obtained a similar chylomicrographic response after feeding, respectively, olive oil and oleic acid to rats.

We now wish to report the results of some experiments in which [1-^{14}C]oleic acid has been fed either in free form or as triolein to rats with a thoracic-duct fistula. Some observations on the distribution of the labelled acid in these two forms to rats without a fistula have also been made.

EXPERIMENTAL

Methyl [1-^{14}C]oleate and [1-^{14}C]oleic acid were prepared according to Bergström, Pääbo & Rottenberg (1952), and triolein was prepared from oleic acid via the acid chloride prepared with oxalyl chloride.

The labelled substances were fed to rats with a thoracic-duct fistula (*A*) and also to animals without a fistula (*B*). The specific activity of the [1-^{14}C]oleic acid was 2300 counts/min./mg. (10 $\mu\mu$c/mg.).

A. *Animals with a thoracic-duct fistula*

Male rats weighing about 250 g. and starved for 12 hr. before operation were used. The procedure used for cannulating the thoracic duct was, with a slight modification, that described by Bollman *et al.* (1948). Instead of a straight cannula, a heated polythene cannula was bent at right angles about 0·5 cm. from the tip and was inserted through the left lateral body wall into the thoracic duct. This bend permits a cannula of larger calibre to be used, as the tension on the tube after insertion is reduced. After operation the rats were placed in restraining cages and had free access to 1% (w/v) NaCl solution until an adequate lymph flow was obtained. Thereafter, 0·64% (w/v) NaCl solution with 0·04% (w/v) KCl was fed *ad lib.* during the whole experiment. Three hours before the labelled substance was administered the rat had free access to white, fat-free bread. These rats were given either 0·5 ml. of labelled oleic acid or triolein by stomach tube when under light ether anaesthesia.

The lymph was collected for 24 hr., after which the animals were killed. In most of these experiments the rat in its cage was placed in a metabolic chamber and the expired CO_2 was recovered in NaOH. The lymph fat was extracted with ethanol–ether (3:1, v/v) and after the evaporation of this extract *in vacuo* the residue was extracted with light petroleum (b.p. 40–60°)–$CHCl_3$ (2:1, v/v). The total fat from the lymph samples was separated into neutral fat and phospholipids on columns of silicic acid and these fractions were saponified as described by Borgström (1952*c*).

The whole gastrointestinal tract was removed in one piece and digested, together with the faeces collected during the experiment, with a solution of 30% (w/v) KOH at 100° for 12 hr. The mixture was then acidified and the fatty acids were extracted with light petroleum (b.p. 40–60°). The amount of fat absorbed in these experiments was calculated by taking the difference between the amount of isotope administered and the amount recovered from the intestinal tract and faeces.

The small activity present in the intestinal wall has thus been included in the unabsorbed amount in these cases, but this amount is negligible in fistula animals 24 hr. after feeding.

B. *Non-cannulated rats*

The labelled material was administered by gastric intubation in amounts corresponding to 0·1 ml./sq.dm. body surface (Lee, 1929). For comparison with the cannulated animals the activity of the expired CO_2 was measured on some rats which had received 0·5 ml. labelled material through a stomach tube. At different intervals after the feeding the rats were bled to death under ether anaesthesia. The gastrointestinal tract was removed and the contents of the small intestine were washed out with saline.

The small intestine was finely dispersed in ethanol in a Waring Blendor and the total fat extracted with ethanol–ether (3:1, v/v), and after the evaporation of this extract *in vacuo* the residue was extracted with light petroleum (b.p. 40–60°)–$CHCl_3$ (2:1, v/v). The total fat was then fractionated into neutral fat and phospholipids on silicic acid columns and saponified (Borgström, 1952*c*).

Measurement of radioactivity

The determination of ^{14}C in fatty acids was done with a thin end-window Geiger-Müller tube on samples of 'infinite thinness'. Samples of an ethanolic solution were evaporated on aluminium planchets to leave less than 0·2 mg./cm^2.

In order to relate the activity of the fatty acids and the activity of the expired CO_2 that was counted as $BaCO_3$ at 'infinite thickness', suitably diluted samples were plated as described above and similar samples were oxidized to CO_2 and counted as $BaCO_3$ at 'infinite thickness' (Bergström, Borgström & Rottenberg, 1951).

RESULTS

The results of the lymph experiments (*A*) are given in Table 1. When labelled oleic acid was fed as the free acid, 66–92% (mean 78·2%) of the fed activity was absorbed in 24 hr. Rats receiving labelled triolein absorbed 57–92% (mean 76·2%) of the activity. It is seen that the fed free oleic acid was absorbed just as well as triolein in 24 hr. After feeding free [1-^{14}C]oleic acid, 38–96% of the absorbed activity was recovered in the lymph fat from the thoracic duct (mean 63·0%). The corresponding figures obtained when labelled triolein was fed were 51–83% (mean 65·5%). Feeding the labelled oleic acid as the free acid or as the glyceride made no significant difference to the recovery of the activity in the lymph lipids.

About 2% of the activity found in the lymph lipids was in the form of phospholipids, the remainder in the form of neutral fat in both groups. It was found that the labelled fatty acid in the lymph lipids followed the mono-unsaturated fatty acid within the limits of the fractionation method of

Table 1. *Recovery of [^{14}C]oleic acid and [^{14}C]triolein in thoracic lymph after oral administration to rats*

Rat no.	Substance fed	Amount of administered activity absorbed (%)	Amount of absorbed activity recovered in lymph as fatty acids (%)	Amount of lymph fat activity found in: Glyceride fatty acids (%)	Amount of lymph fat activity found in: Phospholipid fatty acids (%)	Amount of absorbed activity in expired CO_2 (%)
1	Oleic acid	91·5	64·0	98·2	1·8	4·8
2	Oleic acid	65·5	38·1	98·8	1·2	3·7
3	Oleic acid	73·0	96·4	97·4	2·6	3·5
4	Oleic acid	91·8	62·5	97·9	2·1	—
5	Oleic acid	69·0	53·8	97·5	2·5	4·5
	Mean	78·2	63·0	—	—	—
6	Triolein	92·1	59·3	97·5	2·5	4·4
7	Triolein	56·5	50·5	97·7	2·3	5·0
8	Triolein	85·0	69·2	97·4	2·6	4·0
9	Triolein	71·0	82·9	98·8	1·2	—
	Mean	76·2	65·5	—	—	—

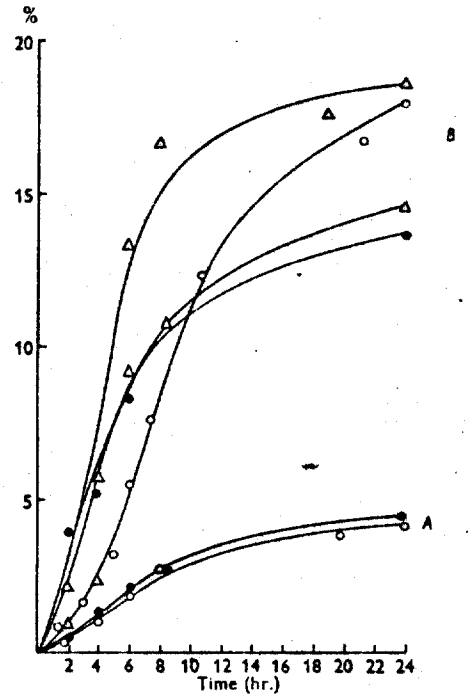

Fig. 1. Percentage of absorbed isotope in expired CO_2 after feeding [^{14}C]oleic acid as triolein (●), methyl oleate (△) or free oleic acid (○). *A*, rats with lymph fistula; every point represents the mean of three animals. *B*, rats without fistula; each point represents a single animal.

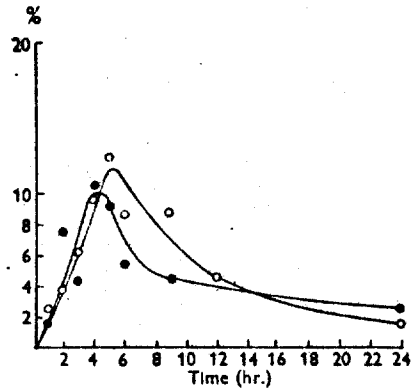

Fig. 2. Relative specific activity of phospholipid fatty acids of small intestine after feeding [^{14}C]oleic acid (○), or [^{14}C]triolein (●).

Bergström & Pääbo (1954). This method is based on chromatographic separation of the products of hydroxylation of the mixture of saturated and unsaturated fatty acid with performic acid.

While non-cannulated rats fed [1-^{14}C]oleic acid or methyl [1-^{14}C]oleate expired about 16% of the absorbed activity as carbon dioxide within 24 hr., rats with the thoracic duct cannulated expired in the same time about 4% irrespective of whether the oleic acid had been administered as glyceride or as free acid (Fig. 1).

In Fig. 2 are given the curves for the specific activities of the fatty acids of the phospholipids of the intestinal wall as percentage of the specific activity of the fed fatty acids. The specific activities of the phospholipid fatty acids increased to a maximum of about 10% of that of the fed fatty acids about 4 hr. after administration. However, only a part of these phospholipids represents material that had been present in the epithelial cells of the mucosa.

DISCUSSION

After feeding labelled free oleic acid or triolein to rats with the thoracic duct cannulated, means of 63·0 and 65·5% of the absorbed activity were

recovered in the lymph lipids during the following 24 hr.

These figures give for the first time quantitative information about the route of transport of oleic acid after absorption from the small intestine.

In corresponding experiments with [^{14}C]palmitic acid dissolved in olive oil (Blomstrand, 1954), in which the same procedures were used as in the experiments reported here, a mean of 67·4 % of the absorbed activity was recovered in the thoracic duct lymph as [^{14}C]fatty acid (range 58·5–75·0 in five experiments). If these figures obtained for labelled palmitic acid are compared with those obtained for oleic acid in this investigation no significant differences are found.

The figures obtained for palmitic acid recovered in the lymph in these experiments are not as high as those given by Bloom *et al.* (1950). These authors found a mean of 75·9 % of the absorbed activity in the thoracic duct lymph as [^{14}C]fatty acid (range 58·5–91·8 in ten experiments). The differences of the means in these two series of experiments may be due to differences in the analytical methods used. Bloom *et al.* (1950) determined the radioactivity directly from the total fatty acids of the lymph obtained after saponification, while in experiments from this laboratory the neutral and phospholipid fatty acids were isolated by a chromatographic procedure before the activity determinations. In this way small losses of activity probably occurred.

Probably there are several causes for the large variation between different animals. There are certainly considerable anatomical differences between different animals in the arrangement of lymph vessels, some of which might by-pass the fistula. Possibly anastomoses exist between lymph and blood vessels which make possible an increased by-passing of the thoracic duct if the lymph flow is partly blocked by, for instance, clots that form in the lymph vessels of some cannulated animals. Also the lymph flow is probably not entirely normal with an open fistula. It is not unlikely that the rats with the highest recoveries are those most approaching normal.

It is seen in Fig. 1, however, that the amount of isotope expired during 24 hr. by a cannulated rat is roughly one-fourth to one-third of the amount expired by the non-cannulated rat. This corresponds roughly to the loss of labelled material via the fistula and shows that the absorbed labelled compounds are metabolized at a normal rate. It must be emphasized that the extent of lymphatic transport of oleic acid was found to be the same as palmitic acid when studied with the same technique (Blomstrand, 1954). No difference was observed in the recovery of labelled oleic acid in the thoracic-duct lymph lipids during the 24 hr. following the administration of labelled oleic acid as free acid or as triolein. The capacity of the intestinal cells to synthesize triglycerides is thus apparently very great. These findings are in general in agreement with the results earlier obtained on the absorption of long-chain unsaturated fatty acids in humans, dogs and rats by Munk (1880), Munk & Rosenstein (1891), Freeman & Ivy (1935), Bollman *et al.* (1950) and Fernandes (1953).

Frazer (1943) followed the absorption of oleic acid and olive oil in the rat with chylomicron counting and reached other conclusions. In the case of olive oil he observed a quicker and stronger response, which led him to the interpretation that the free oleic acid fed was absorbed directly via the portal route and rapidly metabolized in the liver, whereas the olive oil was mainly absorbed via the lymphatics and deposited in the fat depot. These interpretations formed part of the basis for the original 'fat-fatty acid partition theory'. Chylomicron counting—a method of doubtful quantitative significance—seems unsuitable for studies of this kind and has led to erroneous interpretations of this phase of the absorption process (for further discussion cf. Bergström & Borgström, 1954).

That fatty acids of different structure are incorporated into the phospholipids of the small intestinal wall to different extents has been found in earlier work with elaidic acid (Sinclair & Smith, 1937), conjugated linoleic acid (Barnes, Miller & Burr, 1941), and with different saturated fatty acids (Bergström *et al.* 1952; Borgström, 1952*d*).

The values obtained for the incorporation of labelled oleic acid in this work correspond to those found with double-bond conjugated linoleic and myristic acids.

In conclusion it must be emphasized that a very great variability in the rate of the absorption of fat and fatty acids is to be expected. Not only is the general condition of the animal of importance but the presence and nature of other food constituents greatly affect the absorption rate. The influence of these factors on the route of absorption and distribution in the body seems to be of less importance.

SUMMARY

1. The intestinal absorption of [1-^{14}C]oleic acid has been studied in rats with cannulated thoracic ducts.

2. It was found that oleic acid was transported via the thoracic-duct lymph and incorporated into triglycerides and phospholipids to the same extent as found for palmitic acid in earlier work. The same results were obtained whether the oleic acid was fed as free acid or as triolein.

3. The incorporation of the labelled acid into the lipids of the intestinal wall and the appearance of the isotope in the expired carbon dioxide has been followed.

4. The results indicate clearly that there is no detectable difference between the routes followed during absorption of fed free oleic or palmitic acids, or of glycerides containing these acids.

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PARTICIPATION OF PHOSPHOLIPIDES IN LYMPHATIC TRANSPORT OF ABSORBED FATTY ACIDS*

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Recently, we investigated the path by which an absorbed, long chain fatty acid leaves the intestine (1). Unanesthetized rats, into whose thoracic ducts or lacteals cannulae had been introduced, were fed C^{14}-labeled palmitic acid as either the triglyceride or the free acid. From 70 to 92 per cent of the absorbed, labeled fatty acid was recovered as fatty acid C^{14} from the thoracic duct lymph in nine of ten rats studied, and from 69 to 84 per cent was recovered from the intestinal lymph of four rats. The amounts recovered in *intestinal lymph* in the present investigation (Table II) were as high as 97 per cent. Such high recoveries indicate that the transport of absorbed, long chain fatty acids is a concern, almost exclusively, of the intestinal lacteals.

Plasma and lymph serve as avenues of transport between organs and tissues. The fatty acid transport function of plasma phospholipide was dealt with in an earlier report from this laboratory (2). When C^{14}-labeled palmitic acid was injected into fasted, liverless dogs, significant amounts of the labeled fatty acid were incorporated into the phospholipide of the small intestine and its mucosa, the kidneys, lungs, heart, and skeletal muscles. But practically none of the isotopic fatty acids was recovered in the plasma phospholipides. This finding lends no support to the view that *plasma* phospholipides serve to transport fatty acids between organs and tissues.

The present study was designed to test the participation of *lymph* phospholipides in carrying absorbed fatty acids from the small intestine to the plasma.

EXPERIMENTAL

Treatment of Rats

Collection of Lymph—Male rats of the Long-Evans strain were used. They were fed a diet composed of 67.5 per cent whole wheat, 15 per cent

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cascin, 10 per cent whole milk powder, 5.2 per cent hydrogenated vegetable oil, 1.5 per cent calcium carbonate, 0.75 per cent sodium chloride, and 16 cc. of sardilene (a fish oil rich in vitamins A and D) per kilo. The rats were *not* fasted before the operation. Lymph was collected from unanesthetized rats kept in restraining cages (3).

After recovery from the anesthetic, which was induced for the purpose of inserting the cannulae into the lymphatics, the animals had free access to the diet noted above and 1 per cent NaCl solution. The NaCl, by increasing the rate of lymph flow (4), reduces clot formation in the cannula. Once a free flow of lymph was established, the NaCl solution was replaced by tap water (except for two rats; see Table I).

Administration of Labeled Fatty Acids—Palmitic acid, labeled with C^{14} at the carboxyl position, was synthesized (5). Approximately 4 mg. of its triglyceride were dissolved in 0.5 cc. of corn oil. The mixture was then brought to body temperature and administered by intragastric intubation to lightly etherized rats.

Analytical Procedures

Lipides were extracted from each lymph sample and dissolved in petroleum ether (Extract A) as described earlier (1). Separate aliquots of this extract were used to determine the following: fatty acid C^{14} (1), phospholipide fatty acids, total fatty acids, and total cholesterol.

Phospholipide Fatty Acids—The phosphorus content of Extract A was determined by King's method (6). Phospholipide phosphorus was converted to total phospholipide by the factor 25, and the latter to phospholipide fatty acids by the factor 0.68.

Total Fatty Acids and Total Cholesterol—Aliquots of the petroleum ether Extract A were saponified and acidified, and the lipides were dissolved in petroleum ether. Fatty acids were measured by the oxidative procedure (7), and cholesterol was determined by the colorimetric method of Sperry and Brand (8).

Separation of Acetone-Soluble from Acetone-Insoluble Lipides—Aliquots of Extract A were transferred to 50 cc. centrifuge tubes, and the solvent was evaporated to approximately 0.5 cc. The phospholipides were precipitated by the addition of 30 cc. of acetone and 15 drops of a saturated solution of $MgCl_2$ in absolute alcohol. The mixture was centrifuged, and the supernatant containing the acetone-soluble lipides was decanted. 30 cc. of acetone were now added to the precipitate, and the latter was thoroughly dispersed by vigorous stirring. The treatment with acetone was repeated twice more. The four supernatants were then combined; this *acetone-soluble fraction* contained the non-phospholipide fatty acids and cholesterol.

The acetone-soluble and insoluble fractions were used for the determination of non-phospholipide fatty acid C^{14} and phospholipide-fatty acid C^{14}, respectively.

The *acetone-insoluble fraction* (phospholipides) was saponified as follows: The precipitate was dissolved in methanol and to this was added 0.5 cc. of 90 per cent KOH. The mixture was refluxed for 1 hour on a steam bath, cooled, and acidified. The fatty acids were extracted by four washings with hot petroleum ether.

The *acetone-soluble fraction* was likewise saponified and acidified, and its fatty acids were extracted with four separate portions of petroleum ether. It has previously been shown that the cholesterol in the lymph collected under the conditions of this experiment does not contain detectable quantities of C^{14} (1).

The C^{14} contents of these fractions were measured by the direct mounting technique of Entenman *et al.* (9).

Lipide Analysis of Liver and of Feces Plus Gastrointestinal Contents—At the end of the experiment the animals were sacrificed and the gastrointestinal contents and feces, excreted from the time of administration of the labeled fat, were analyzed for lipide C^{14} as described in an earlier report (1), which also deals with the determination of the lipide C^{14} in the liver.

Results

Seven rats were studied. In three cannulae were introduced into the intestinal lymphatics, and in four, into the thoracic ducts. As a rule, rats so treated begin to drink about 6 hours after the operation, and to eat within 12 hours. After clotting had ceased to occur in the cannulae and lymph was flowing freely, the labeled triglyceride was administered by stomach tube. The interval between the insertion of the cannula and feeding of the test fat was about 25 hours (Table I). The animals had access to food throughout the entire period of observation. Rats 6 and 7 received 1 per cent NaCl for drinking purposes during the period of lymph collection. The other five rats received the saline for the first 18 to 24 hours and tap water thereafter.

Under the influence of the saline the rats excreted about 2 cc. of lymph per hour (Table I). The substitution of tap water reduced the rate to about 1 cc. per hour. This latter value is in agreement with the rates reported by Bollman *et al.* for the 200 gm. rat (10).

Lipides of Lymph—The lipide composition of the two types of lymph is recorded in Table I. There was no apparent difference between the lipide composition of the intestinal lymph and that of the thoracic duct lymph obtained from rats fed fat.

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0.7 to 1.6 gm. of fatty acids was recovered from the total lymph collected in 20 to 22 hours from thoracic duct or intestinal lacteals. This amount of fatty acids far exceeds that administered by stomach tube (less than 0.5 gm.), but this was to be expected since the rats had access to food during the experiment.

In the fed, *unanesthetized* rat, only about 7 per cent of the total fatty acids in either thoracic duct or lacteal lymph was phospholipide fatty acids. In the case of the anesthetized rabbit, Frölicher and Süllmann (11) reported that 7 to 13 per cent of the fatty acids in intestinal lymph

TABLE I

Flow Rates and Lipide Composition of Intestinal and Thoracic Duct Lymph Obtained from Rats

Rat No.*	Lymph collected from	Total amount of lymph collected: Before fat administration		Total amount of lymph collected: After fat administration		1 per cent NaCl solution replaced by tap water after	Composition of lymph collected after feeding labeled fat†: Total cholesterol	Total fatty acids recovered‡	Phospholipide fatty acids: Amount recovered‡	Phospholipide fatty acids: Per cent of total fatty acids
		hrs.	*cc.*	*hrs.*	*cc.*	*hrs.*	*mg. per cent*	*mg.*	*mg.*	
1	Lacteals	25	43	22	22.5	24	64.2	1000	73.7	7.4
2	"	25	49	22	25.4	23	62.1	1096	78.2	7.1
3	"	24	61	22	31.2	22	69.2	1572	108.2	6.9
4	Thoracic duct	29	79	20.5	22.2	23	59.0	876	57.7	6.6
5	" "	27	74	20	18.1	18	62.4	956	63.5	6.6
6	" "	27	29	20	57.5		23.3	742	57.5	7.8
7	" "	22	21	21	34.3		55.4	1460	95.0	6.5

* 210 to 300 gm. in weight.

† The stock diet was available to all rats throughout the experiment.

‡ The intervals during which these amounts were recovered are recorded in the fifth column.

is present as phospholipide fatty acids. Since Reinhardt *et al.* (12) have shown that, in the dog, plasma phospholipides are transferred to thoracic duct lymph, it is not possible to state how much of the phospholipide fatty acids recovered from intestinal lymph in our experiments was derived from ingested fat.

Lymph contained from 55 to 69 mg. per cent of cholesterol in six of the seven rats studied.

C^{14}-Labeled Lipides in Lymph—The percentages of the administered labeled fat absorbed are recorded in Table II. The term *absorbed* as used here refers to the difference between the amount of C^{14}-labeled fat

administered and that recovered from the gastrointestinal tract and feces at the end of the experiment. In five of the seven rats studied, 70 to 83 per cent of the *administered* palmitic acid C^{14} was absorbed. Rats 1 and 7 absorbed 41 and 53 per cent, respectively. The low absorption in Rat 1 probably resulted from an intestinal disturbance, for its stools became liquid after the administration of the test fat. Table II also shows, in confirmation of our earlier findings (1), that nearly all of the absorbed C^{14} can be recovered in the fatty acid fraction of lymph obtained from either the thoracic duct or intestinal lymphatics. In five of the seven rats, 90

TABLE II

Fatty Acid C^{14} Recovered in Lymph after Enteral Administration of Palmitic Acid-1-C^{14}

The palmitic acid labeled in the carboxyl position with C^{14} was administered as the tripalmitin dissolved in 0.5 cc. of corn oil at body temperature by intragastric intubation. Each rat received approximately 4 mg. of radioactive tripalmitin containing 1×10^5 c.p.m. per mg.

Rat No.	Per cent of administered palmitic acid C^{14} absorbed*	Per cent of absorbed palmitic acid C^{14} recovered: In lymph as fatty acid C^{14}	Per cent of absorbed palmitic acid C^{14} recovered: In liver as lipide C^{14}	Per cent of lymph fatty acid C^{14} recovered in: Phospholipide fraction	Per cent of lymph fatty acid C^{14} recovered in: Non-phospholipide fraction
1†	40.8	81.4	0.75	3.7	96.3
2	77.5	95.7	0.50	3.3	96.7
3	82.2	96.7	0.75	3.6	96.4
4	83.3	92.8	0.36	2.3	97.7
5	77.5	90.7	0.55	3.0	97.0
6	70.3	63.3	0.36	3.7	96.3
7	52.7	96.5	0.51	3.1	96.9

* Absorbed refers to the difference between the amount administered and the amount recovered from the feces and intestinal contents.

† This rat developed diarrhea after the administration of the labeled fat.

per cent or more of the *absorbed* C^{14} was present as fatty acid C^{14} in the collected lymph.

Not more than 4 per cent of the C^{14}-labeled fatty acids recovered from both intestinal and thoracic duct lymph had been incorporated into phospholipides.

DISCUSSION

The finding that 96 per cent of the labeled fatty acids in lymph was present in non-phospholipide form again emphasizes the unimportance of phospholipides as vehicles for fatty acid transport. It is also of interest to note that very little of the lymph fatty acids is carried in the form of cholesterol esters. Even if we assume that all of the cholesterol in lymph

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is esterified, carriage in this form could account for, at most, 1.5 per cent of the non-phospholipide fatty acids contained in lymph. It would appear from these observations, as well as from those made earlier by Goldman *et al.* (2), that fatty acids are transported chiefly in forms other than phospholipides.

The results obtained also bear on the site of formation of plasma phospholipides. Our earlier studies with *fasted*, liverless dogs indicate strongly that the liver is the chief site for the formation of plasma phospholipide (2, 13, 14). This was shown in experiments carried out with P^{32} and with C^{14}-labeled palmitic acid. But the significance of the small intestine as a contributor to plasma phospholipides during fat absorption was not satisfactorily settled. The present findings show that, during the absorption of fat, it is possible for intestinal lymph to contribute phospholipides to plasma. Although the indications are strong that the intestinal lymph phospholipide containing the C^{14}-fatty acids was synthesized in the small intestine, the possibility that it was formed in part elsewhere (liver or mesenteric lymph nodes) is, of course, not ruled out. The extent to which the small intestine serves as an extrahepatic site for plasma phospholipide formation during fat absorption is being investigated.

SUMMARY

1. The present study was undertaken to test the participation of lymph phospholipides as vehicles for the transport of an absorbed, long chain fatty acid from the small intestine to plasma. Palmitic acid, the carboxyl carbon of which was labeled with C^{14}, was fed to rats in which cannulae had been introduced into the intestinal lymphatics or thoracic ducts. As much as 96 per cent of the C^{14}-labeled fatty acids recovered in the lymph obtained from these two sources was present in forms other than phospholipides. The conclusion is drawn that phospholipides are not important transport forms for absorbed palmitic acid.

2. The contribution of the small intestine to the synthesis of plasma phospholipides during fat absorption is discussed.

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THE QUANTITATIVE SIGNIFICANCE OF THE LYMPHATIC PATHWAY IN TRANSPORT OF ABSORBED FATTY ACIDS*

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The claim that the major portion of absorbed fat passes into the lymph stream is based on the classical experiment of Munk and Rosenstein carried out more than 50 years ago on a patient suffering from elephantiasis, in whom a lymphatic fistula developed in the left leg (1). From such a fistula, these workers were able to recover as much as 60 per cent of the fat of an ingested meal. Such a high recovery was not observed by later investigators in animal experiments. Eckstein, working with dogs fed emulsified olive oil, reported recoveries in thoracic duct lymph as high as 21 per cent in 12 hours, and 17 per cent in 6 hours (2). When he fed the dogs oleic and palmitic acids, the recoveries were much lower. Since the dogs were anesthetized throughout the entire period of lymph collection, Eckstein was careful to point out that he did not expect fat absorption to be as good as it would have been under more normal conditions. Such low recoveries of absorbed fat in thoracic duct lymph of the dog were also observed by Little and Robinson (3).

The availability of C^{14}-labeled fatty acids, together with the development by Bollman *et al.* (4) of a method for collecting lymph from the *unanesthetized rat*, made possible a reinvestigation of this problem.

EXPERIMENTAL

Collection of Lymph

Male rats of the Long-Evans strain, not fasted before the operation, were used in this study. The procedures used for obtaining samples from the thoracic duct and intestinal lacteals were modifications of those described by Bollman, Cain, and Grindlay (4).

Thoracic Duct—The peritoneal cavity was opened by a mid-line incision extending from the xiphisternal junction to a point about 3 cm. from the symphysis pubis. With the aid of the dissecting microscope, the thoracic duct was carefully freed from the dorsal aorta. A polythene cannula was

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inserted through the left lateral body wall just caudal to the costal margin, and introduced into the duct, where it was securely fixed by two ligatures. The muscular wall and skin were then closed by continuous suture. The entire operative procedure was usually completed in 25 minutes, during which the rats were under ether anesthesia.

Intestinal Lymphatic—The two intestinal lymphatic trunks pass along the superior mesenteric artery and are connected by anastomotic vessels. To obtain intestinal lymph, the polythene cannula was inserted into the larger trunk, and the smaller trunk was ligated.

Lymph collection from the unanesthetized animal was made possible by keeping the rat in a restraining cage similar in design to that described by Bollman (5). The rats with thoracic duct fistulae had free access to food and 1 per cent NaCl solution for the duration of the experiment. Those from which intestinal lymph was collected had free access to food throughout. Before administration of the labeled fatty acid, a 1 per cent NaCl solution was available for drinking purposes; thereafter, tap water was made available.

Administration of Labeled Fatty Acids

Palmitic acid labeled with C^{14} at the carboxyl position was synthesized by the method described elsewhere (6). It was administered either as the tripalmitin or as the free fatty acid dissolved in 0.5 cc. of corn oil. The mixtures were brought to body temperature and administered to the rats, which had been lightly anesthetized with ether, by intragastric intubation. The duration of anesthesia at this point did not exceed 5 minutes.

Methods

Measurement of Absorption of Fatty Acids—At the end of the observation period, the rats were sacrificed by an intraperitoneal injection of sodium pentobarbital (8 mg. per 100 gm. of body weight). The stomach was removed, and its contents were carefully transferred to a beaker by washing it with 95 per cent ethyl alcohol. The intestinal tract was severed at the ileocecal junction. A glass tube was then inserted into the duodenal end and the lumen washed with 200 cc. of 95 per cent ethyl alcohol. The contents of the stomach and small intestine were combined with the washings and analyzed for C^{14} in the lipide.

The cecum and large intestine were incised and their contents carefully transferred to a beaker by means of alcohol washings. The feces, collected from the time of administration of the labeled fatty acid, were added to the same beaker. The entire sample so collected was analyzed for C^{14} in the lipide.

Extraction of Lipides

Contents of Stomach and Small Intestine—The alcoholic extract was decanted and the residue thoroughly ground in a mortar. The ground residue was then recombined with the alcoholic extract referred to above and sufficient ethyl ether added to the mixture to yield a solvent consisting of 3 parts of alcohol to 1 part of ether. The mixture was heated for 1 hour at 55°. The extract was decanted and the residue reextracted with about 100 cc. of a 3:1 alcohol-ether mixture. The combined extracts were filtered and the residue was washed three times with freshly distilled ethyl ether. The filtrates and washings were concentrated at 55° under reduced pressure to a volume of approximately 1 cc.; the last 20 to 30 cc. were removed in an atmosphere of CO_2. The concentrate was extracted five times with hot petroleum ether. The petroleum ether extracts were combined and made up to a desired volume.

Contents of Cecum and Large Intestine and Feces—The alcohol was decanted, the residue thoroughly ground, and the latter recombined with the alcoholic extract in the manner described above. The entire mixture was acidified and, after sufficient amounts of alcohol and ether had been added to provide approximately 200 cc. of 3:1 alcohol-ether, it was extracted at 55° for 1 hour. The extract was decanted and the residue reextracted with approximately 100 cc. of 3:1 alcohol-ether. The residue resulting from this second extraction was refluxed overnight with ethyl ether in a Soxhlet apparatus. All the extracts were now combined and concentrated, and a petroleum ether extract of the concentrate was prepared as described above.

Lymph—The lymph samples were extracted with 20 volumes of a 3:1 alcohol-ether mixture at 55° for 1 hour. The supernatant was decanted and the solids were reextracted, at the same temperature, with 100 cc. of this alcohol-ether mixture. A petroleum ether extract was prepared as described above.

Liver—The lipides were extracted from this tissue without acidification in the manner described for the contents of the cecum and large intestine and feces.

Determination of Lipide-C^{14} in Liver and in Contents of Gastrointestinal Tract—Aliquots of the petroleum ether extracts containing total lipides were mounted on aluminum disks for determination of their C^{14} content as described by Entenman *et al.* (7).

Determination of Fatty Acid-C^{14} in Lymph—Aliquots of the petroleum ether extracts were brought almost to dryness, and the residue was saponified by alcoholic potassium hydroxide for 1 hour on a steam bath. The hydrolysate was acidified by the addition of 20 per cent aqueous H_2SO_4

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and then exhaustively extracted in a liquid Soxhlet with petroleum ether. Aliquots of this petroleum ether extract were transferred to aluminum disks and their C^{14} content was determined (7).

In order to express the C^{14} values as fatty acid-C^{14}, it was necessary to determine the C^{14} content in cholesterol in fractions isolated as described above. Petroleum ether solutions of lymph lipides were evaporated to dryness in an atmosphere of CO_2, and the residue was saponified as described above. Sufficient alcohol was added to the saponified lipides to raise the concentration of alcohol in the mixture to 50 per cent. Cholesterol was then removed by exhaustive extraction with petroleum ether. Most of the fatty acids, in the form of their soaps, were removed from the petroleum ether extract by washing three times with normal KOH. The cholesterol was then precipitated with digitonin, and the digitonide was freed of fatty acid contaminants by repeated washings with several solvents. The C^{14} content of the cholesterol digitonide was determined as described by Srere *et al.* (8). In none of the samples tested was C^{14} activity found.

Results

In the early intervals, clot formation interferes with lymph flow. For this reason, the rats were kept under constant observation, and any clots that formed were removed as soon as possible. Clots can be readily removed by insertion of a fine barbed piano wire (0.008 inch in diameter) into the cannula in order to entangle the clot. Mann *et al.* (9) have shown that this clot formation ceases about 18 hours after the establishment of the fistula, a finding that has been confirmed in this laboratory. Therefore, at least 20 hours were allowed to elapse before the administration of the labeled fat. In all the rats used in this study, lymph flow was unobstructed during the period following the administration of the labeled material.

Experiments in Which Thoracic Duct Lymph Was Collected—Ten rats with thoracic duct fistulae were studied (Table I). Four received the labeled palmitic acid in the form of tripalmitin, whereas the other six received it as the free fatty acid. Lymph was collected thereafter for 19 to 24 hours.

The rats were sacrificed 19 to 24 hours after the samples of labeled fatty acid were administered, and at this time nine of the ten rats had absorbed 80 to 95 per cent of the labeled fatty acid. Since negligible amounts of the isotope were found in the contents of the stomach and small intestine, it would appear that ample time had been allowed for absorption from the small intestine.

From 36 to 118 cc. of lymph were collected from the rats after the labeled

TABLE I

Recovery of Enterally Administered Labeled Palmitic Acid in Thoracic Duct Lymph

The palmitic acid used was labeled in the carboxyl position with C^{14}. It was dissolved in 0.5 cc. of corn oil at body temperature and administered by intragastric intubation. Each rat received approximately 4 mg. of labeled palmitic acid containing 1×10^5 counts per minute per mg.

Rat No.	Weight	Total lymph collected: Before fat administration		Total lymph collected: After fat administration		Form of labeled palmitic acid administered	Per cent of administered palmitic acid-C^{14} recovered: In stomach and small intestine contents as lipide-C^{14}	Per cent of administered palmitic acid-C^{14} recovered: In cecum and large intestine contents and feces as lipide-C^{14}	Per cent of administered palmitic acid-C^{14} absorbed	Per cent of absorbed palmitic acid-C^{14} recovered: In lymph as fatty acid-C^{14}	Per cent of absorbed palmitic acid-C^{14} recovered: In liver as lipide-C^{14}
	gm.	hrs.	cc.	hrs.	cc.						
1	200	52	108	20	43	Tripalmitin	2.3	17.2	80.5	85.7	0.34
2	238	28	14	20	36	"	0.5	51.8	47.7	58.3	0.41
3	185	23	42	23	94	"		7.3*	92.7	70.7	0.19
4	287	24.5	38	24	91	"		5.0*	95.0	73.0	0.19
5	200	53	204	19	98	Palmitic acid	1.4	9.8	88.8	70.0	0.35
6	310	27	28	21	101	" "	0.7	6.7	92.6	76.4	0.41
7	386	27.5	49	21	118	" "	2.3	14.5	83.2	78.5	0.26
8	201	21	58	19	78	" "		13.5*	86.5	69.8	0.23
9	183	23	65	19	96	" "		15.5*	84.5	79.7	0.20
10	260	24	12	21	45	" "	0.2	14.2	85.6	91.8	0.25

* The entire gastrointestinal contents were combined for analysis.

TABLE II

Recovery of Enterally Administered Labeled Palmitic Acid in Intestinal Lymph

The palmitic acid used was labeled in the carboxyl position with C^{14}. It was dissolved in 0.5 cc. of corn oil at body temperature and administered by intragastric intubation. Each rat received approximately 4 mg. of labeled palmitic acid containing 1×10^5 counts per minute per mg.

Rat No.	Weight	Total lymph collected: Before fat administration		Total lymph collected: After fat administration		Form of labeled palmitic acid administered	Per cent of administered palmitic acid-C^{14} recovered: In stomach and small intestine contents as lipide-C^{14}	Per cent of administered palmitic acid-C^{14} recovered: In cecum and large intestine contents and feces as lipide-C^{14}	Per cent of administered palmitic acid-C^{14} absorbed	Per cent of absorbed palmitic acid-C^{14} recovered: In lymph as fatty acid-C^{14}	Per cent of absorbed palmitic acid-C^{14} recovered: In liver as lipide-C^{14}
	gm.	hrs.	cc.	hrs.	cc.						
20	260	28	20	24	25	Palmitic acid	0.6	6.6	92.8	83.8	0.17
21	250	23	40	24	46	" "	0.2	64.5	35.3	83.2	0.39
22	350	22	15	25	52	" "	0.3	13.9	85.8	68.7	1.1
23	290	20	5	24	24	" "	3.9	6.1	90.0	74.8	0.53

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fatty acid was administered, or about 2 to 6 cc. per hour. Table I shows the percentages of the *absorbed* C^{14} recovered as fatty acid-C^{14} in lymph. About 58 per cent was recovered in Rat 2. In the other nine rats, from 70 to 92 per cent of the absorbed palmitic acid-C^{14} appeared in the fatty acids of lymph.

Experiments in Which Intestinal Lymph Was Collected—The results of this experiment are recorded in Table II. Three of the four rats studied absorbed 86 to 93 per cent of the labeled palmitic acid introduced into their stomachs. In these rats, from 69 to 84 per cent of the absorbed palmitic acid-C^{14} was recovered in the fatty acid fraction of intestinal lymph.

Despite the fact that only 35 per cent of the administered labeled fatty acid was absorbed by Rat 21 (Table II), the recovery of fatty acid-C^{14} in its intestinal lymph amounted to 83 per cent of that absorbed.

DISCUSSION

In a study involving as complex a process as the transport of absorbed fat by way of the lymphatic stream, it is possible for the condition of the animal to influence that process. For this reason, we must consider the experimental conditions under which thoracic duct lymph was collected. The rats received an anesthetic during insertion of the cannula, less than half an hour, and again at the time that fatty acid was administered, a procedure requiring at most 5 minutes. Insertion of the cannula necessitated a certain amount of intestinal manipulation, but none of the rats was used earlier than 20 hours after insertion of the cannula, and some were not used until 53 hours afterward. Another factor that needs consideration is the loss of lymph before the introduction of the test fat and during the period of lymph collection. It should be stressed that the rats began to drink saline as early as 6 hours after introduction of the cannula and to eat food within 12 hours, and that they continued to do so throughout the entire period of lymph collection. The almost complete absorption of the administered labeled fatty acid also indicates that the experimental procedures had not seriously interfered with the activity of the small intestine.

As much as 92 per cent of the absorbed fatty acids was recovered from the thoracic duct lymph. There can no longer be any doubt that most of the absorbed fat *can* be transported by way of the lymphatic stream. The percentages of the absorbed palmitic acid-C^{14} recovered in lymph were apparently not influenced by the forms in which the labeled fatty acid was administered; *i.e.*, tripalmitin and free palmitic acid. This finding is not consistent with the partition hypothesis of fat absorption proposed by Frazer (10, 11).

Ever since the original experiments, many reviewers have evinced considerable interest in the path of absorption of the 40 per cent of the ingested fat that was not recovered from the lymph fistula of Munk and Rosenstein's patient, and the conclusion has often been drawn that 40 per cent of absorbed fat passes directly into portal blood. The incomplete recoveries of absorbed palmitic acid-C^{14} in the lymph of the rats reported here would also appear to argue in favor of a portal pathway for absorbed fatty acids. But the possibility cannot be excluded that all of the fatty acid-C^{14} entered the lymph stream and that, subsequently, a portion of it, namely that not recovered in the collected lymph, reached the systemic circulation by way of lymphatic-venous anastomoses. Although evidence for the existence of such anastomotic channels has been presented (12–14), their rôle in fat transport has not yet been assessed. For this reason, the question whether any of the fatty acid-C^{14} passes directly into portal blood must be held in abeyance.

Of considerable interest in this connection is the finding of C^{14} activity in the liver of rats in which thoracic duct lymph had been diverted by cannulation. The small amounts of C^{14} present in the liver at the time the animals were sacrificed (19 to 24 hours after the administration of the labeled fatty acids) does not detract from the significance of this observation because of the ease with which hepatic tissue oxidizes higher fatty acids. The possibility that the C^{14} reached the liver directly by way of portal blood or by lymphatic-venous anastomosis has been pointed out above. Another possibility is the degradation of the labeled palmitic acid during its passage through the intestinal wall and the subsequent passage of the C^{14} into the circulation.

SUMMARY

1. A study on the transport of absorbed C^{14}-labeled palmitic acid via thoracic duct and intestinal lymph of *unanesthetized* rats is presented.

2. The rats received palmitic acid-1-C^{14} either in the form of tripalmitin or as the free fatty acid. In 19 to 24 hours, twelve of the fourteen rats studied absorbed from 81 to 95 per cent of the administered fatty acid-C^{14}.

3. From 70 to 92 per cent of the absorbed palmitic acid-C^{14} was recovered as fatty acid-C^{14} from *thoracic duct* lymph in nine of the ten rats studied.

4. From 69 to 84 per cent of the absorbed palmitic acid-C^{14} was recovered as fatty acid-C^{14} from *intestinal* lymph of four rats.

5. The question of transport of absorbed fatty acids by routes other than the lymph stream is discussed.

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STUDIES ON DIASTATIC ACTIVITY

1. THE ACTION OF UNSATURATED FATTY ACIDS AND SOAPS ON THE ENZYME DIASTASE. *

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The work of Jobling and Petersen[1] in 1915 on proteolytic and allied ferments is so important and of such a stimulating character that one is surprised that the outcome has not been more extensive. These observers showed that fatty acids and lipoids containing unsaturated "double bonds" inhibit the action of proteolytic ferments. Lipoids present in human serum exert such an action on the proteases present, and this action can be modified either by removing the lipoids with fat solvents or by saturating their "double bonds", as, for example, with halogen derivatives.

The fact that the fatty acid content of human serum is in a continual state of flux has, strange to say, not been stressed. Bloor[2] found that the ether-soluble constituents of normal plasma before breakfast ranged ordinarily from 0.57 to 0.82 per cent. Considerable variations in these values occur, depending upon the food intake. Thus, the same writer[3] found lipemia only in diabetics receiving fat in their food, the lipemia disappearing under fasting. As this variation must especially affect those substances containing unsaturated bonds, then, provided Jobling and Petersen's work stands, it is natural to conclude that the proteolytic value of serum must vary also. In conditions where there is a great increase in the fat and fatty acid content of the blood, one would expect that the proteolytic activity of the blood would be greatly diminished, and perhaps in the opposite condition, viz: when the fat content was decreased, the proteolytic activity would be increased. Other factors, of course, play a part in the modification of proteolytic activity,

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especially H ion concentration, as is so evident from the researches of Opie[4] and others.

In 1918, Stocks[5] published his paper wherein he extended this work on proteolytic ferments to diastatic phenomena. Using a commercial preparation of pancreatic diastase and the Wohlgemuth technique, Stocks concluded that:

1. "Sodium oleate and other oleates inhibit the action of diastase on starch.
2. This inhibitory reaction exhibits the quantitative characters of a chemical combination between the two substances.
3. The diastatic activity of normal blood serum is also inhibited by oleates.
4. Pure fats and other lipoid substances were not found to inhibit diastase.
5. Inactivated human serum showed no anti-diastatic property."

At the commencement of 1923, while working on the diastatic activity of urine as a test for pancreatic efficiency, I performed somewhat similar experiments to those of Stocks, using olive oil (containing free oleates) and saliva as reagents. My results coincided with those of Stocks but I was not satisfied of their correctness, until I came across the former's paper. Even then there appeared to be some danger in accepting these conclusions. Anyone who has had an extended experience with the Wohlgemuth technique cannot fail to be critical of its accuracy. In most conditions where an increase of diastatic activity in a fluid, such as blood or urine, is very definite, as, for example, in pancreatitis, this test is of real value. Speaking paradoxically, its quantitative results are of a qualitative value only. The end point with iodine is extraordinarily indefinite in many cases, while with very active diastatic solutions, unless one uses a large number of tubes, the values vary tremendously. I therefore decided to investigate the subject with more exact and easily controllable methods.

As the result of my experiments, I am able to confirm the work of Stocks as regards the oleate-diastase reaction, although I have not attempted to extend it to human serum.

The present position thus appears to be: Lipins, especially fatty acids and their derivatives, containing unsaturated "double bonds" have a definite inhibitory action on diastatic ferments.

Perhaps one of the most interesting results obtained in an extensive series of diastatic estimations of urine and blood is that the value is either normal or subnormal in diabetes mellitus. In the course of some five hundred estimations, I have examined the urine of sixteen diabetics for diastase, and in each have found values between the limits of two and twenty-five units (normal limit, ten to thirty units). On consulting the literature, I find that this has been recognised by several observers. Harrison and Lawrence[16] in a recent study found that both the concentration and excretion of diastase were subnormal in a certain number of diabetics. Allen[6] concluded that diastatic estimations were of little value in diabetes mellitus, presumably on these grounds. Now, in diabetes mellitus, it is generally recognised that lipemia is a common, if not a constant, finding. The masterly work of Allen and his associates, Bloor and many others, may be mentioned in passing. Gray[7] states that in this lipemia the glycerides and fatty acids are chiefly increased, many of which contain unsaturated bonds. Is it possible that, knowing the action of such substances on diastatic ferments, we have an explanation of the low diastatic value of blood and urine in diabetes mellitus? One does not necessarily suggest that this is the only factor concerned, for questions of H ion concentration, dilution of urine, etc. must be considered, but at least it is of some value as a basis for further work.

Now, in most cases of pancreatic disease, with the exception of malignancy, the diastatic activity of blood and urine is greatly increased and in many of these, especially when the Islets of Langerhans are not diseased, there is no lipemia. Are we justified in summing up as follows:

Pancreatic disease {inflamatory / obstructive} No lipemia

High diastatic value of blood and urine.

Diabetes mellitus—Lipemia

Low diastatic value.

Should this be so, it is only a small step forward to suggest that blood diastatic and fat values may run side by side, and that the former may be of some prognostic importance. Certainly

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with limited laboratory facilities, the blood diastatic test could be carried out very easily.

Various other problems arise, amongst which may be mentioned the influence of unsaturated fatty acids and fats on duodenal and intestinal digestion. Under certain conditions, this type of lipin is present in fairly large proportions in the duodenum and consequently must exert an inhibitory effect on the enzymes present, this effect increasing in proportion to the increase of lipins. Hence as digestion proceeds, the activity of the enzymes — proteolytic and diastatic — tends to decrease.

But there are several compensatory mechanisms at work, viz: the activating effect of bile, at any rate on diastase, as will be described in a later communication, and in all probability on the proteolytic ferments, and the constant absorption of fatty acids. One would like to know whether there is an activation of the ferment maltase side by side with the inhibition of diastase, due to these unsaturated lipins.

From a practical point of view, it is evident that administration of extracts of pancreatic diastase, e. g., taka-diastase, must fail or be considerably decreased in value, in the presence of fats or fatty acids of the above type.

In the following experiments salivary amylopsin has been the enzyme basis, being typical in its action of the various diastases found throughout the human body. Oleic acid and sodium oleate provided the necessary unsaturated substances, being easily obtained and convenient to work with.

METHOD

General outline: The method used is similar to that described by C. L. Evans[8] in 1912. A fixed quantity of diluted saliva, obtained from a healthy male, is allowed to act on a certain quantity of starch solution, in the presence of a sodium oleate or oleic acid solution. With each experiment, a control test in which distilled water is substituted for the oleate solution is carried out under exactly the same conditions and with identical quantities of reagents. Both mixtures are then titrated with Tenth Normal Sodium Hydroxide, using "Neutral Red" as indicator, until a faint pink tinge is obtained, giving a pH of approximately 6.4.

Samples of the mixtures are removed at ten minutes intervals

and, using the Mohr-Bertrand method, the copper-reducing power of each sample is obtained, thus giving an exact idea of the activity of the enzyme at the moment of withdrawal of the sample. Curves are obtained by plotting the time of withdrawal of the samples against the copper-reducing power of the samples, i. e., the measure of enzyme activity, and clearly show the whole process of digestion. The following experiments will be described:

1. The action of saliva on a starch solution, under constant conditions.
2. Preliminary determination of the influence of sodium oleate on the curve given by saliva acting on a starch solution.
3. The time-factor in the action of sodium oleate on diastase.
4. The concentration-factor in the action of sodium oleate on diastase.
5. The temperature factor in the action of sodium oleate on diastase.
6. Alterations due to saturation of sodium oleate.
7. The effect of adding boiled sodium oleate solution to a diastatic solution.
8. The effect of adding starch solution to sodium oleate for a fixed period before carrying out a digestion experiment.

DETAILS:

The diastatic solution was in every instance fresh human Saliva, obtained from a healthy male aet, 24 years and diluted ten times with distilled water.

A freshly prepared starch solution, in some cases 2% strength, in others 1% strength, in distilled water was used in 100 cc. and 120 cc. quantities.

Sodium oleate solution, freshly prepared with distilled water, was used in strengths varying from 1 in 10,000 to 1 in 10.

Thus a typical digestion mixture consisted of:

Starch, 2% solution.....	120 cc.
Saliva, diluted 1 in 10.....	10 "
Sodium oleate, 1 in 100.....	10 "

and the corresponding control would be:

Starch, 2% solution.....	120 cc.
Saliva, diluted 1 in 10.....	10 "
Distilled water	10 "

Titration of both mixtures was then carried out as described above.

Such a mixture would be placed in a flask of 250 cc. capacity and allowed to remain at the required temperature in a water-bath for fifty minutes,

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20 cc. samples being withdrawn at ten minutes intervals and the enzymic activity stopped by adding 0.5 cc. of strong ammonia.

Usually the starch solution was brought to the temperature of the water-bath before the other solutions were added. The samples, after 20 cc. of Bertrand's No. (1) and (2) solutions had been added, were immediately heated to boiling point for exactly three minutes, allowed to stand until the precipitate settled at the bottom of the flask, and the supernatant fluid filtered through an asbestos filter with filter-pump attached. The precipitate, after being washed with warm water, was dissolved in 10 to 20 cc. of Bertrand's No. (3) reagent, the whole being filtered through the same asbestos filter so as to dissolve any retained precipitate. The filtrate was then titrated with Bertrand's No. (4) reagent, the number of cc. of this determining the copper-reducing power of the samples. By means of Bertrands' and Thomas' tables the amount of sugar in the sample can be obtained, but in such an investigation as the present, nothing is to be gained by such figures. All that is required is an accurate measure of the enzyme activity at certain time intervals. This method is rapid, easy of performance, and sufficiently accurate for all purposes, especially where, as in this investigation, the controls are rigid and results are based on comparisons.

The only fallacy which one is able to determine is that there is a reaction between potassium permanganate, contained in Bertrand's No. (4) solution, and sodium oleate, with reduction of the former. Test experiments under the same conditions as in the following show that if the process of titration be carried out rapidly this is of little importance, amounting to no more than one or two percentages of error. Where differences between control and test experiments are small, then this source of error may be of some importance, but throughout this investigation, quantities of reagents and the conditions have been so planned as to give well-defined results.

RESULTS:

(1) *The action of saliva on a starch solution*

The following experiment is typical:

The digestion mixture consisted of

Starch, 2% solution	120 cc.
Saliva, diluted 1 in 10	10 "
Distilled water	10 "

Titrated as described above.

Incubated at 46°C., samples (20 cc.) withdrawn every ten minutes for fifty minutes.

Time	Bertrand No. 4 reagent.
10 minutes	7.75 cc.
20 "	10.4 "
30 "	12.1 "
40 "	12.7 "
50 "	13.0 "

The curve obtained by plotting time against copper-reducing

power (in cc. of Bertrand's No. 4 reagent) is shown in Figure 1 It will be noticed that equilibrium point is very nearly reach in forty minutes.

Variations in strength of saliva, concentration of starch solu tion and temperature produce modifications in such a curve, bu the form is always constant.

(2) *Preliminary determination of the influence of sodium oleate on th curve obtained as in Section* (1)

The following experiment is characteristic:
120 cc. starch solution, 2% strength, plus 10 cc. Saliva of dilution 1 in 10 were incubated at a temperature of 46°C. along with:

A.	10 cc. distilled water	Control
B.	10 cc. sodium oleate	1 in 10,000 solution
C.	10 cc. sodium oleate	1 in 1,000 solution
D.	10 cc. sodium oleate	1 in 100 solution

Titration being carried out as described above.

Subsequent procedures were as described above, and curves were ob tained as in Figures 1 and 2.

RESULTS:

A. Control, with distilled water. See Figure 1.

Time	Bertrand No. 4 reagent.
10 minutes	7.75 cc.
20 "	10.4 "
30 "	12.1 "
40 "	12.7 "
50 "	13.0 "

B. Sodium oleate, 1 in 10,000. See Figure 2.

Time	Bertrand No. 4 reagent.
10 minutes	7.2 cc.
20 "	10.2 "
30 "	11.7 "
40 "	12.5 "
50 "	12.7 "

C. Sodium oleate, 1 in 1,000. See Figure 2.

Time	Bertrand No. 4 reagent.
10 minutes	6.75 cc.
20 "	9.75 "
30 "	11.35 "
40 "	11.8 "
50 "	11.9 "

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D. Sodium oleate, 1 in 100. See Figure 2.

Time	Bertrand No. 4 reagent.
10 minutes	2.4 cc.
20 "	3.6 "
30 "	4.6 "
40 "	5.5 "
50 "	5.7 "

This series of experiments shows conclusively that oleates have a definite inhibitory effect on diastase, thus confirming Stocks' statement. Similar results, though less marked, were obtained by using oleic acid instead of its sodium salt. The experiments also demonstrated that a 1 in 100 solution of sodium oleate is a very convenient solution to work with, the inhibitory power being marked, yet not too great to be easily determined by the above method.

Of the various other experiments of similar nature, the results were the same. An attempt was then made to find out more about this oleate-diastase reaction, and the following series of experiments was worked out.

(3) *The time factor in the action of sodium oleate on diastase.*

In these experiments an attempt was made to determine whether the relationship between sodium oleate and diastase was influenced by a time factor. Accordingly, sodium oleate solutions were added to a diastatic solution for varying time periods, and the action of the latter on starch was then determined as in previous experiments.

The details are as follows: 10 cc. of sodium oleate, 1 in 100 solution, plus 10 cc. of saliva, diluted 1 in 10, were mixed, and then added to 100 cc. of starch, 2% solution:

A. Immediately after mixing.
B. One hour after mixing.
C. Two hours after mixing.
D. Four hours after mixing.
E. Six hours after mixing.

Each mixture was at once titrated as in previous experiment.

RESULTS:

A. Immediately on mixing.

Time	Bertrand No. 4 reagent.
10 minutes	4.9 cc.
20 "	7.3 "
30 "	8.9 "
40 "	10.0 "

B. One hour after mixing.

Time	Bertrand No. 4 reagent.
10 minutes	2.3 cc.
20 "	3.7 "
30 "	4.9 "
40 "	5.5 "

C. Two hours after mixing.

Time	Bertrand No. 4 reagent.
10 minutes	1.2 cc.
20 "	2.1 "
30 "	2.8 "
40 "	3.3 "

D. Four hours after mixing.

Time	Bertrand No. 4 reagent.
10 minutes	0.7 cc.
20 "	1.4 "
30 "	1.9 "
40 "	2.1 "

E. Six hours after mixing

Time	Bertrand No. 4 reagent.
10 minutes	0.6 cc.
20 "	1.1 "
30 "	1.5 "
40 "	1.8 "

Control:

Time	Bertrand No. 4 reagent.
10 minutes	10.0 cc.
20 "	14.8 "
30 "	17.1 "
40 "	18.5 "

The time factor is shown very well by plotting the enzyme activity at forty minutes after commencement of digestion against the time period during which the sodium oleate and diastase were in contact, i.e. the number of cc. of Bertrand's No. 4 solution reduced by the sample of the starch digest obtained forty minutes after the commencement of the experiment against the number of hours during which the diastase and sodium oleate had been together. This is shown in Figure (4).

Details of other experiments along similar lines are not set out here for the sake of brevity, but in all cases confirmatory results were obtained.

That a definite relationship exists between the time of diges-

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tion and the period during which the sodium oleate and the diastase have been in contact, the effect being kept constant, is seen from an examination of the following table, derived from Figure (3).

Effect = e.	Time of Digestion = t hours.	Period of contact between Sodium Oleate and Diastase = p hours.	$t \times \frac{1}{p}$
0.86 cc. Bertrand No. 4...	0.056	1	0.056
" " ...	0.11	2	0.055
" " ...	0.2	4	0.05
" " ...	0.29	6	0.048
1.43 cc. " ...	0.096	1	0.096
" " ...	0.23	2	0.115
" " ...	0.37	4	0.093
" " ...	0.55	6	0.092

Within a small limit of effect, viz: that toward which the curve in Figure (4) converges, then the product of the time of digestion and the inverse of the period during which the sodium oleate and the diastase have been in contact remains a constant, provided the effect be kept constant, i.e. $t \times \frac{1}{p} = k$, when e is constant.

(4) *The concentration factor in the action of sodium oleate on diastase.*

In these experiments, the concentration of the sodium oleate was varied, the amount of enzyme remaining constant. Starch digests were carried out in the usual manner, the oleate and enzyme solutions being added at once to the starch, so that the time-interval was kept constant. The following experiment may be taken as typical of several.

100 cc. starch, 2% solution, was added to 10 cc. saliva, diluted 1 in 10, and 10 cc. of sodium oleate solution of the following concentration was added to this mixture:

(a)	Sodium oleate.....	1 in 1000 solution
(b)	" "	1 in 500 "
(c)	" "	1 in 250 "
(d)	" "	1 in 100 "
(e)	" "	1 in 50 "
(f)	" "	1 in 25 "
(g)	" "	1 in 10 "

Each mixture being titrated as described above.

Results were plotted as in previous experiments. The temperature

throughout was 37°C. In order to economize space, the amount of digestion occurring in forty minutes is shown, and a graph obtained (Figure 5) by plotting the concentration of sodium oleate against the amount of digestion occurring in forty minutes.

Concentration of sodium oleate	Time of digestion	Bertrand's No. 4 reagent
1 in 1000	40 minutes	16.4 cc.
1 in 500	"	15.0 "
1 in 250	"	13.6 "
1 in 100	"	8.9 "
1 in 50	"	3.5 "
1 in 25	"	2.5 "
1 in 10	"	2.0 "

By a method similar to that used in the preceeding section, it can be shown that within certain small limits the product of the time of digestion and the inverse of the concentration of sodium oleate is a constant, when the effect is kept constant.

(5) *The temperature factor in the action of sodium oleate on diastase.*

In these experiments, the temperature at which sodium oleate was allowed to act on saliva, was varied, the concentration of sodium oleate being kept constant. As one was dependent upon the ordinary laboratory facilities, which did not include a thermostat, for this work, it is with a certain amount of hesitation that the following results are presented, and it is preferred to give the facts only without drawing any conclusions.

100 cc. starch, 1% solution, plus 10 cc. saliva, diluted 1 in 10 and 10 cc. sodium oleate, 1 in 100 solution, were mixed, titrated, and placed in a water bath at the following temperatures:

Expt. A	Mixture incubated at.....	16.5°C.
" B	" " "	28.0°C.
" C	" " "	36.5°C.

Controls were carried out with distilled water in place of sodium oleate solution, at each temperature.

Experiment A - 16.5°C.	Time	Bertrand's No. 4 reagent
Oleate added.	10 minutes	1.4 cc.
	20 "	2.5 "
	30 "	3.2 "
	40 "	3.5 "
Control.	10 minutes	4.0 cc.
	20 "	6.6 "
	30 "	8.5 "
	40 "	9.8 "

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	Time	Bertrand's No. 4 reagent
Experiment B - 28.0°C. Oleate added.	10 minutes	1.5 cc.
	20 "	2.6 "
	30 "	3.1 "
	40 "	3.6 "
Control.	10 minutes	5.0 cc.
	20 "	8.2 "
	30 "	10.6 "
	40 "	11.4 "
Experiment C - 36.5°C. Oleate added.	10 minutes	1.6 cc.
	20 "	2.6 "
	30 "	3.3 "
	40 "	3.7 "
Control.	10 minutes	6.7 cc.
	20 "	9.8 "
	30 "	11.1 "
	40 "	12.0 "

In another experiment of similar nature, the following results were obtained:

Temperature.		Time	Bertrand's No. 4 reagent
16.5°C.	Oleate added	40 minutes	2.3 cc.
	Control	"	6.8 "
26.0°C.	Oleate added	40 minutes	2.6 cc.
	Control	"	7.4 "
34.5°C.	Oleate added	40 minutes	2.4 cc.
	Control	"	7.9 "
46.6°C.	Oleate added	40 minutes	2.6 cc.
	Control	"	8.6 "

In an experiment in which the reacting substances were each first carefully brought to the temperature of experimentation before being mixed, similar results were obtained. One would like very much to investigate further this problem under the most rigid conditions.

(6) *Alterations due to saturation of sodium oleate.*

Sodium oleate was treated with iodine (0.5 gm. iodine to 50 cc. sodium oleate, 1% solution, with a few crystals of potassium iodide) overnight. Free iodine was then removed by shaking the iodised oleate solution with chloroform until the latter was clear, as in the method described by Jobling and Petersen.[8] An iodised oleate solution was thus obtained reasonably purified as regards free iodine.

The following mixtures were allowed to digest:

A.	Starch, 2% solution	100 cc.
	Saliva, diluted 1 in 10	10 "
	Sodium oleate, 1% solution	10 "
B.	Starch, 2% solution	100 cc.
	Saliva, diluted 1 in 10	10 "
	Iodised sodium oleate, 1% solution	10 "
C.	Starch, 2% solution	100 cc.
	Saliva, diluted 1 in 10	10 "
	Distilled water	10 "

Temperature of experiments, 40°. Titration similar.

RESULTS:

	Time	Bertrand's No. 4 reagent
A. Sodium oleate only	10 minutes	4.8 cc.
	20 "	7.5 "
	30 "	9.2 "
	40 "	10.0 "
B. Iodised sodium oleate	10 minutes	8.0 cc.
	20 "	14.4 "
	30 "	16.4 "
	40 "	17.0 "
C. Control	10 minutes	8.8 cc.
	20 "	15.4 "
	30 "	17.6 "
	40 "	18.2 "

It will be seen from Figure (6) that, allowing for the presence of free iodine which had not been removed by the chloroform, saturation of sodium oleate removes its inhibitory action on diastase, thus bringing this enzyme into line with the proteolytic enzymes, as regards their relationship to sodium oleate.

That iodine has a very definite inhibitory effect on diastase is clearly shown in the following experiment:

100 cc. starch, 2% solution, plus 10 cc. saliva, diluted 1 in 10, plus 10 cc. iodised distilled water (i. e. water treated with iodine, as described above) were allowed to incubate at 40°C. A control experiment under identical conditions, using distilled water in the place of iodised water, was carried out.

RESULTS:

	Time	Bertrand's No. 4 reagent
Iodised water	10 minutes	8.0 cc.
	20 "	12.4 "
	30 "	14.4 "
	40 "	15.0 "

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Water only	10 minutes	8.5 cc.
	20 "	15.0 "
	30 "	17.4 "
	40 "	18.0 "

This inhibitory effect of iodine can be shown to vary directly with the concentration of iodine present.

(7) *The effect of adding boiled sodium oleate solution to a diastatic solution.*

The following experiment demonstrates that boiling sodium oleate has no effect on its inhibitory action.

The digests were:

A.	Starch, 2% solution	100 cc.
	Saliva, diluted 1 in 10	10 "
	Sodium oleate, 1% solution, boiled for 15 minutes and then cooled	10 "
B.	Starch, 2% solution	100 cc.
	Saliva, diluted 1 in 10	10 "
	Sodium oleate, 1% solution	10 "

Temperature of experiments, 40°. Titration similar.

RESULTS:

	Time	Bertrand's No. 4 reagent
(A) Boiled sodium oleate	10 minutes	2.6 cc.
	20 "	5.0 "
	30 "	6.3 "
	40 "	6.9 "
(B) Control	10 minutes	3.0 cc.
	20 "	5.8 "
	30 "	7.0 "
	40 "	7.8 "

Within the ordinary limits of error, the results are very similar.

8) *The effect of adding starch solution to sodium oleate for a fixed period before carrying out a digestion experiment.*

In these experiments, the starch solution and the sodium oleate were mixed, titrated, and placed in the incubator at 37°C. for twenty hours. The saliva was then added, and a curve of digestion obtained as in previous experiment (Figure 7).

The mixtures used were:

(A)	Sodium oleate, 1% solution	10 cc.
	Starch, 2% solution	100 "
		incubated for 20 hours at 37°C.
	Saliva, diluted 1 in 10	10 "
		then added.
(B)	Sodium oleate, 1% solution	10 cc.
	Starch, 2% solution	100 "
	Saliva, diluted 1 in 10	10 "
		mixed at once.

RESULTS:

	Time	Bertrand No. 4 reagent.
(A)	10 minutes	5.5 cc.
	20 "	7.8 "
	30 "	9.4 "
	40 "	9.8 "
(B)	10 minutes	2.0 cc.
	20 "	3.6 "
	30 "	4.6 "
	40 "	5.3 "

The mixture of starch and sodium oleate which had been incubated for 20 hours was also tested for diastase, before saliva was added, by the Wohlgemuth technique, but none was present. It appears then that the starch solution has so acted on the sodium oleate that the latter has lost part of its inhibitory power.

DISCUSSION:

Although the method used throughout this investigation has several disadvantages, it is claimed that it presents a much more accurate account of the conditions of diastatic activity than the ordinary Wohlgemuth method as used by Stocks. It is therefore extremely interesting to find that the work of this observer, as regards the action of sodium oleate on diastase, is confirmed. The enzyme diastase may thus be said to obey the same rule that Jobling and Petersen first put forward in regard to proteolytic enzyme.

Several general conclusions as to the oleate-diastase reaction may be drawn. First, that within certain small limits, provided the effect be kept constant, the product of the time of digestion and the inverse of the period during which the sodium oleate and

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the diastase are in contact remains a constant. Second, that within certain small limits, provided the effect is kept constant, the product of the time of digestion and the inverse of the concentration of sodium oleate remains a constant. These results are not surprising when we remember the general law which has been shown to apply to trypsin by Bayliss[10], to diastase by Brown and Glindinning[11], to peptone splitting ferments by Vernon[12], to invertase by O'Sullivan and Tompson[13] and to fibrin ferments in the venoms of snakes by C. J. Martin[14], viz: that if the effect is constant, then, within certain small limits, the product of the time of digestion into the amount of enzyme is constant. In the case of the time period of action of sodium oleate on diastase, it appears that within certain limits the longer sodium oleate acts on diastase, the less active the latter becomes, and vice versa, so that the inverse of the period during which the sodium oleate and the diastase are in contact really represents the activity of the enzyme, and the first conclusion becomes identical with the general enzyme law.

In the case of the concentration factor, much the same conditions prevail, for the greater the concentration of sodium oleate, the less active the diastase becomes, so that the inverse of the concentration really represents the activity of the enzyme, and once again we are dealing with the general enzyme law.

The alteration due to saturation of sodium oleate in the activity of this substance is in accord with Jobling and Petersen's findings in the case of proteolytic enzymes, and is suggestive of a chemical, rather than a physical, interaction, dependent upon the "double bonds" present in the sodium oleate. The absence of any alteration in this property consequent upon boiling the solution is also suggestive. The action of starch solution upon oleates appears to be paralleled in a statement of Jobling and Petersen[15] that unsaturated lipoids can be absorbed from guinea-pig serum by starch, agar and bacteria. Possibly the oleate-diastase reaction is one of those vague phenomena classified as physico-chemical. Before drawing any definite conclusion as to the nature of this reaction, one would like to see a detailed investigation of the temperature factor carried out under the most exact conditions. That there is some underlying relationship between the temperature and the oleate-diastase reaction seems fairly certain. Only the most careful experimentation can determine this.

SUMMARY:

The action of oleates on the enzyme diastase has been studied, and Stocks' work confirmed, thus bringing amylolytic enzymes into line with proteolytic ferments as regards their relationship to oleates. A time-factor and concentration-factor seem evident. The temperature-factor needs elucidation. Saturation of the oleates, and the effect of boiling are suggestive of a chemical reaction rather than physical. Some evidence is presented that there is also a relationship between starch and oleates, possibly of a physical nature.

ACKNOWLEDGMENTS:

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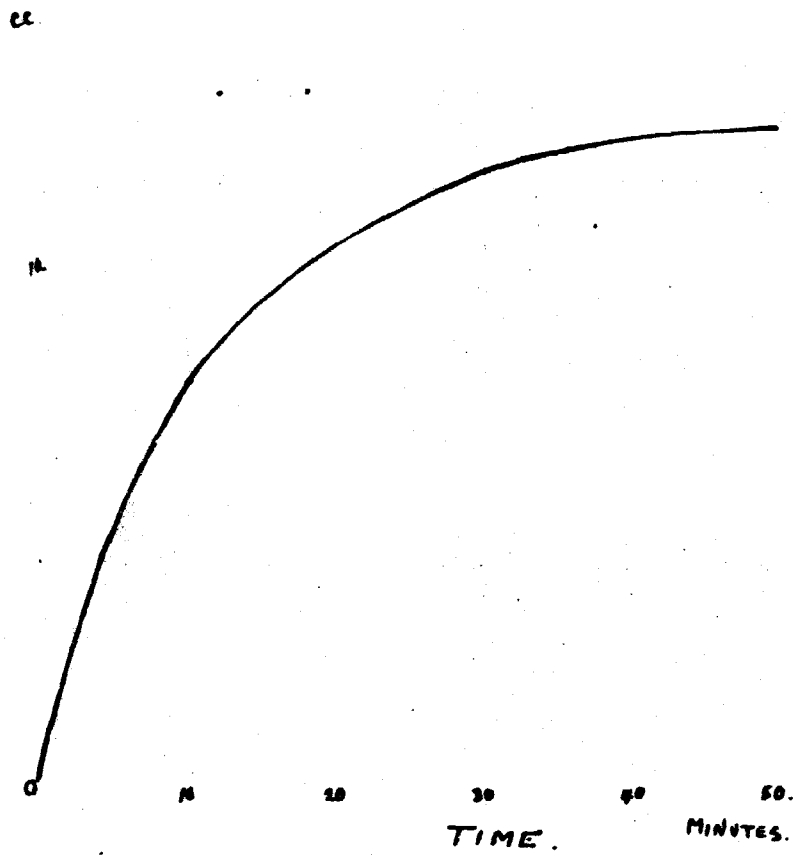

FIG. 1.

The action of Saliva on a starch solution.
Time, expressed in minutes, is plotted against the number of cubic centimetres of Bertrand's No. 4 reagent. Temperature of experiment, 46°C.

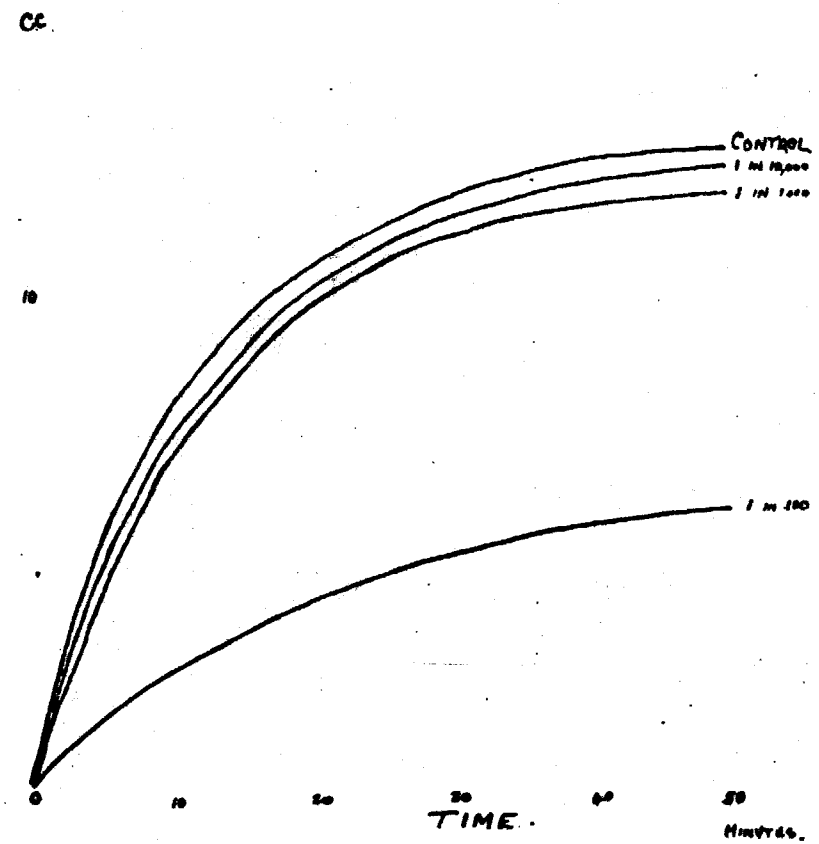

FIG. 2.

The action of Sodium Oleate (1 in 10,000 solutions) on a diastatic solution.
(1 in 1,000 ")
(1 in 100 ")
Time, expressed in minutes, is plotted against the number of cubic centimetres of Bertrand's No. 3 reagent. Temperature of experiments, 46°C.

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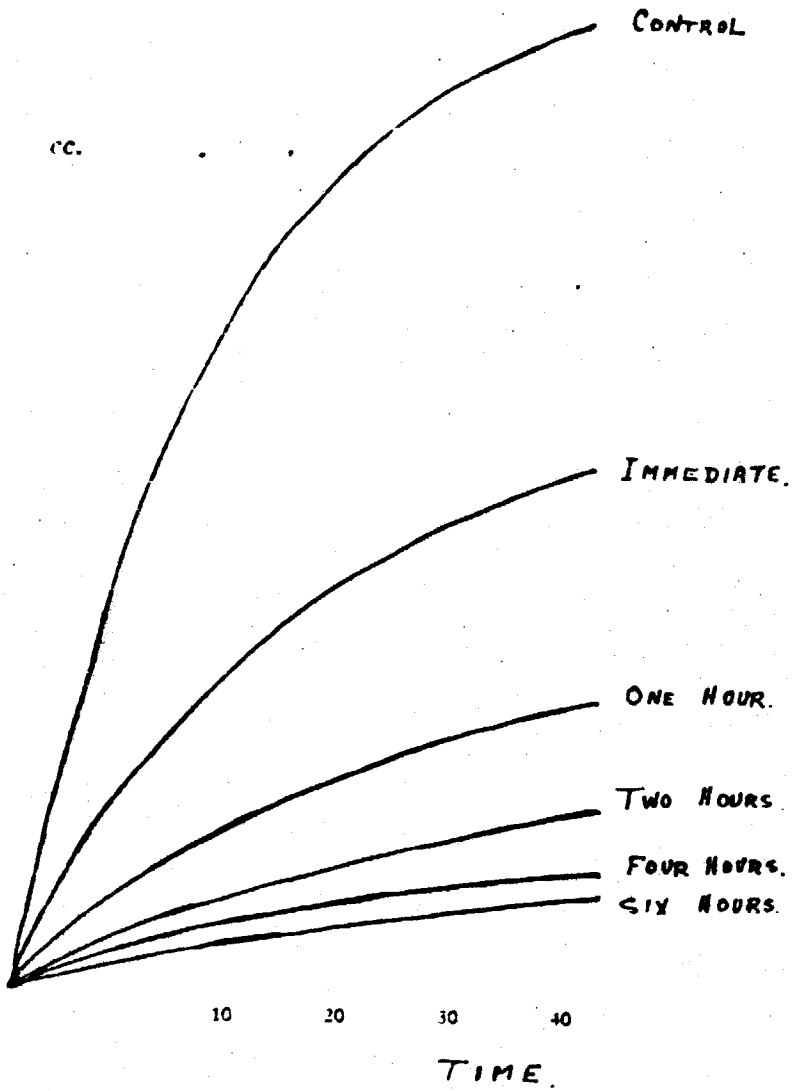

FIG. 3.

The Time Factor in the action of sodium oleate on diastase. Separate curves for time intervals shown. Time plotted against Bertrand's No. 4 reagent. Temperature of experiments, 37°C.

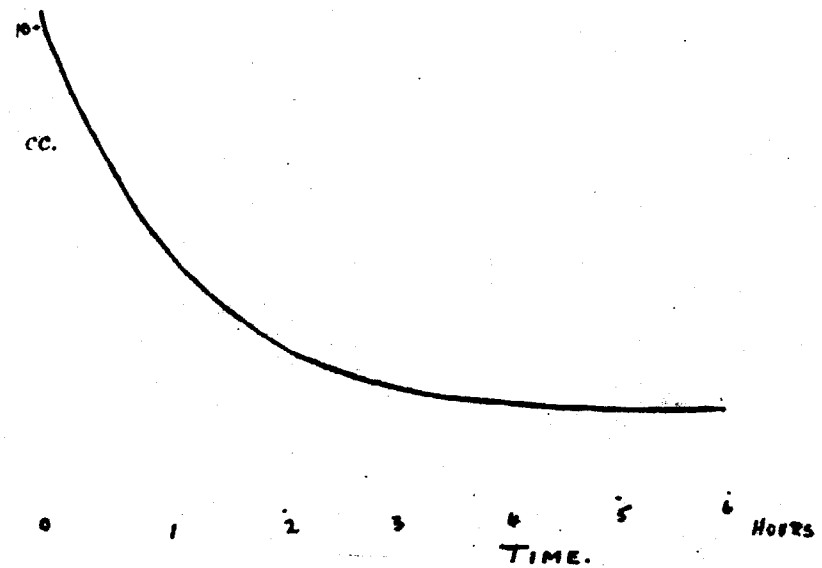

FIG. 4.

The Time Factor in the action of sodium oleate on diastase.

The period during which the sodium oleate and diastase were in contact plotted against the number of cubic centimetres of Bertrand's No. 4 reagent reduced by the sample of the starch digest obtained forty minutes after commencement of experiment.

Temperature of experiments, 37°C.

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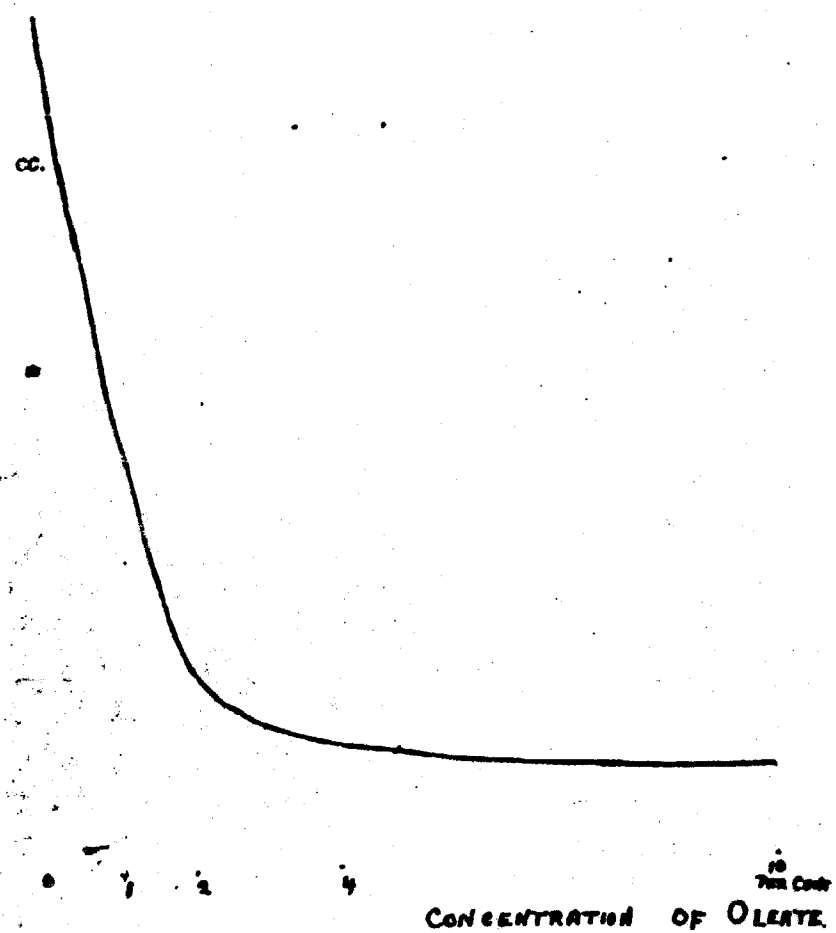

FIG. 5.

The Concentration Factor in the action of sodium oleate on diastase.

Concentration of sodium oleate is plotted against the number of cubic centimetres of Bertrand's No. 4 reagent which is reduced by the sample obtained at the end of forty minutes.

Temperature of experiments, 37°C.

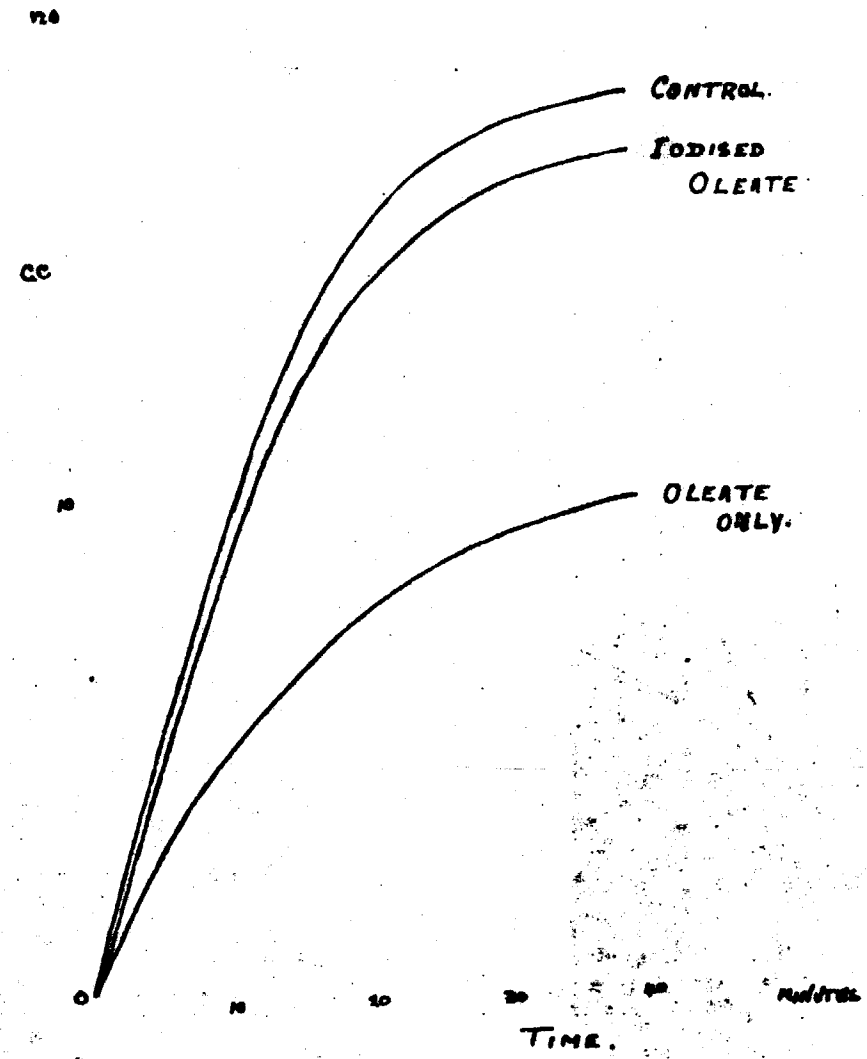

FIG. 6.

Saturation of Sodium Oleate with Iodine.

Showing the effect of sodium oleate only, iodised sodium oleate and the control on the diastatic solution.

Time, in minutes, is plotted against number of cubic centimetres of Bertrand's No. 4 reagent reduced by the digest samples.

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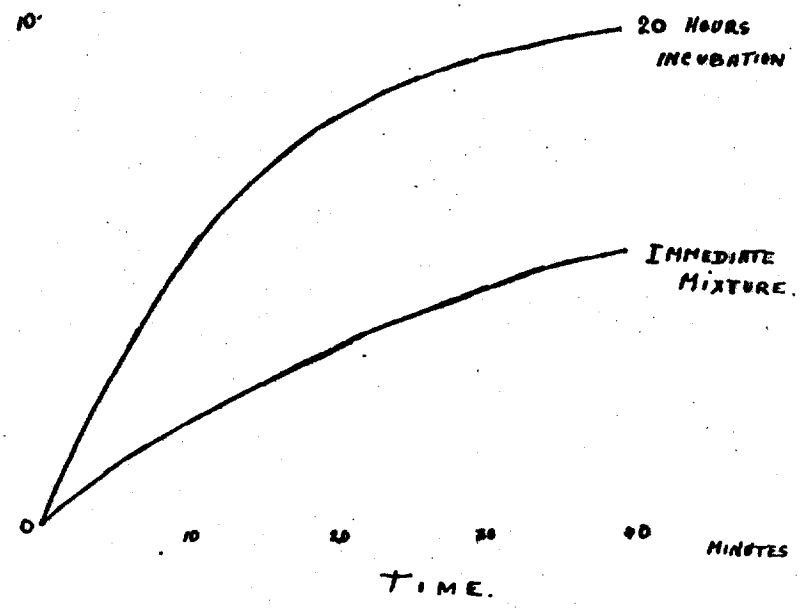

FIG. 7.

Effect of adding Starch solution to Sodium Oleate for a fixed period before carrying out a digestion experiment.

Time is plotted against quantity in cubic centimetres of Bertrand's No. 4

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141:1197-1198, 1947.

Neutralization of neuromuscular effects of dietary lipid imbalance by nicotinamide, pantothenic acid and adermine

by

Paul Chauchard, Henriette Mazoué and Raoul Lecoq

Because of the speed of their intestinal absorption, castor oil, sodium salts of fatty acids and fatty acids have proved to be the cause of dietary imbalance when present in the diet (which includes a normally sufficient dose of the B vitamins given in the form of brewer's yeast)(1). This imbalance, however, observed with any proportion of fatty acids or salts of fatty acids, is only manifested with large quantities of castor oil (40-50%), while lower doses (20-22%) have no effect.

In young rats, such dietary lipid imbalance causes changes in neuromuscular excitability, more or less rapdily manifested by a drop in nerve chronaxy, which persists after anesthesia (encephalomedullary excitation effect), later followed by a rise in muscular chromaxy, an effect which is analogous to that of repeated subcutaneous injections of these same substances (2).

The decline in nerve chromaxy takes place in 4-6 days under the effect of diet containing a high porportion of castor oil and which no carbohydrate in the diet; the decline is considerably delayed, however (10-15 days) in the presence of starch. Sodium oleate and stearic acid, introduced into the diet in a quantity of 20-22% reveal their effects in 48 hours. On the other hand

olive oil (in any proportion, if the diet is otherwise well balanced) and castor oil (in a 20-22% proportion that does not cause imbalance) do not cause any appreciable neuromuscular disturbance within one month.

It was then investigated whether vitamins that play the role of coenzymes for the dehydrogenase effect on higher fatty acids [nicotinamide, pantothenic acid and adernine (pyridoxine), Champougny and Le Breton (3)], whose corrective effect we had noted on neuromuscular disturbances caused by repeated injection of castor oil or salts of fatty acids, might also be capable of neutralizing the effects of unbalanced lipid diets by ensuring correct metabolism.

This was found to be the case: with a daily dose of 100 γ of one factor in the case of oleate, or 200 γ in the case of stearic acid the unbalancing effect is apparently intensified when the fatty acids are not combined with salt, so that there is a rapid return to normal chronaxy. An analogous correction takes place in the case of diets rich in castor oil.

In all cases the omission of vitamins essential to lipid metabolism is quickly followed by a reappearance of chronaxial disturbances.

Conclusions

The usual non-unbalancing lipids, of which olive oil is taken as a typical example, do not necessitate special vitamin intake when the diet containing them includes a normally sufficient quantity of B vitamins. A high proportion of castor

oil in the diet (40-50%), of 20% sodium oleate or stearic acid necessiates the addition to the diet of one or the other of the vitamins that play a role in the metabolism of higher fatty acids: nicotinamide, pantothenic acid or adermine. In doses of 20-22%, castor oil behaves like olive oil. The disturbance is delayed by the inclusion of starch in the diet rich in castor oil.

Bibliography

See footnote references.

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Compte Rend. Soc. Biol. 141 (1947)

P. 1197

Neutralisation des effets neuromusculaires des déséquilibres alimentaires lipidiques par la nicotinamide, l'acide pantothénique et l'adermine.

par Paul Chauchard, Henriette Mazoué et Raoul Lecoq.

En raison de leur rapidité d'absorption intestinale, l'huile de ricin, les sels sodiques d'acides gras et les acides gras se montrent, quand ils sont présents dans un régime (comportant par ailleurs une dose normalement suffisante de vitamines B données sous forme de levure de bière), causes de déséquilibre alimentaire (1). Toutefois ce déséquilibre, qui s'observe pour toutes les proportions d'acides ou de sels d'acides gras, ne se manifeste qu'avec de fortes proportions d'huile

(4*) *C. R. de l'Acad. des sc.*, 1934, t. *198*, p. 1540.
(1) R. Lecoq. Thèse Doct. Sc. nat. Paris, 1934 et *Journ. Pharm. Chim.*, 1937, t. *26*, p. 56.

de ricin (40 à 50 p. 100), des doses plus faibles (20 à 22 p. 100) étant sans action.

De tels déséquilibres alimentaires lipidiques entrainent, chez le jeune Rat, des modifications de l'excitabilité neuromusculaire se traduisant plus ou moins rapidement par une chute des chronaxies nerveuses qui subsiste après anesthésie (action excitante encéphalo-médullaire), puis une augmentation des chronaxies musculaires, action analogue à celle des injections sous-cutanées répétées des mêmes substances (2).

La diminution des chronaxies nerveuses se réalise en 4 à 6 jours sous l'effet des régimes à forte proportion d'huile de ricin en l'absence de glucides dans la ration ; mais cette chute se trouve retardée considérablement (10 à 15 jours) en présence d'amidon. L'oléate de sodium comme l'acide stéarique introduits dans le régime à raison de 20 à 22 p. 100 manifestent leurs effets en 48 heures. Par contre l'huile d'olive (en toutes proportions, si la ration est par ailleurs bien équilibrée) et l'huile de ricin (en proportion de 20 à 22 p. 100 non déséquilibrante) n'amènent en un mois aucune perturbation neuromusculaire sensible.

Nous avons alors recherché si les vitamines qui jouent le rôle de coenzymes permettant l'action de la déhydrase sur les acides gras supérieurs [nicotinamide, acide pantothénique et adermine ou pyridoxine : Champougny et Le Breton (3)] vitamines dont nous avions noté l'action correctrice vis-à-vis des troubles neuromusculaires par injections répétées d'huile de ricin ou de sels d'acides gras, seraient susceptibles également de neutraliser les effets des régimes lipidiques déséquilibrés en assurant un métabolisme correct.

Il en est bien ainsi : avec une dose quotidienne de 100 γ de l'un des facteurs dans le cas de l'oléate, ou de 200 γ dans le cas de l'acide stéarique, l'action déséquilibrante se trouvant, semble-t-il, plus accentuée quand les acides gras ne sont pas salifiés, on obtient un rapide retour des chronaxies à la normale. Une correction analogue se réalise dans le cas des régimes riches en huile de ricin.

Dans tous les cas, la suppression des vitamines indispensables au métabolisme lipidique est rapidement suivie d'une réapparition des troubles chronaxiques.

Conclusions. — Les corps gras habituels non déséquilibrants, dont l'huile d'olive est prise ici comme type, n'entraînent pas le besoin d'apports vitaminiques spéciaux, lorsque le régime qui les contient renferme une proportion normalement suffisante de vitamines B. L'huile de ricin en forte proportion dans la ration (40 à 50 p. 100), l'oléate de sodium et l'acide stéarique dans la proportion de 20 p. 100, nécessitent l'adjonction au régime de l'une ou l'autre des vitamines intervenant dans le métabolisme des acides gras supérieurs : nicotinamide, acide pantothénique, ou adermine. Aux doses de 20 à 22 p. 100, l'huile de ricin se comporte comme l'huile d'olive. Les troubles se trouvent retardés par incorporation d'amidon dans le régime riche en huile de ricin.

(*Laboratoires de neurophysiologie, Hautes-Etudes et de l'hôpital de Saint-Germain-en-Laye*).

(2) *C. R. de la Soc. de biol.*, 1947, t. *141*, p. 1196.
(3) *C. R. de la Soc. de biol.*, 1947, t. *141*, p. 43.

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Stimulation of Insulin Secretion by Infusion of Free Fatty Acids

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ABSTRACT The acute elevation of plasma free fatty acid (FFA) levels by direct infusion of sodium oleate into the plasma of conscious dogs was accompanied by the rapid onset of a 2- to 12-fold increase in plasma immunoreactive insulin, and, subsequently, a marked fall in plasma glucose, even in dogs receiving intravenous glucose throughout the infusion. The magnitude of both the insulin and glucose responses correlated with the mean FFA level during infusion. A large increase in plasma insulin and fall in glucose also occurred when glycerol was infused with oleate in order to simulate endogenous lipolysis more closely. Insulin levels in pancreaticoduodenal vein blood rose markedly during oleate infusion, while plasma ketone levels rose only slightly.

In contrast to the effects of oleate infusion, elevation of plasma FFA to correspondingly high levels by triolein ingestion and intravenous heparin produced only small increases in plasma insulin, which did not correlate well with the FFA level reached, and small increases in plasma glucose.

The results indicate that under certain conditions, elevated FFA levels may be a potent stimulus of insulin secretion. This response is modified under other conditions such as during chylomicron removal under the influence of heparin. This effect may play a role in the regulation of lipolysis and ketone formation, but determination of the exact mechanism of FFA stimulation of the pancreas and its physiological significance will require further investigation.

Preliminary reports of part of this work have appeared (1) and were presented at the Annual Meeting of the Council on Arteriosclerosis, American Heart Association, Bal Harbour, Fla. 20 November 1968 (2).

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INTRODUCTION

Preliminary studies in this laboratory (3) showed that a marked decrease in blood glucose occurred during acute elevation of plasma free fatty acids (FFA) levels by infusion of sodium oleate directly into the plasma of conscious dogs. Subsequently, we found that a striking elevation of plasma insulin levels accompanied the glucose response (1). At that time, Seyffert and Madison (4) independently reported that the acute elevation of plasma FFA levels by intravenous infusion of triglyceride emulsion and heparin also was accompanied by a rise in plasma insulin and fall in glucose in anesthetized dogs with chronic portacaval shunts.

In contrast, Schalch and Kipnis (5) and Balasse and Ooms (6) have reported that elevation of plasma FFA by fat ingestion and intravenous heparin did not significantly affect basal insulin and glucose levels in man. In the present study, the effects of FFA infusion on insulin secretion and blood glucose levels were determined and then compared to those accompanying administration of a fat meal and heparin.

METHODS

FFA infusions. The method of elevating plasma FFA levels in dogs by direct infusion of FFA salts during continuous-flow centrifugation has been described in detail (7).

Mongrel dogs of both sexes were studied after an overnight fast. Except where noted anesthesia was not employed. Carotid artery blood was continuously separated into cells and plasma at a constant rate of 150–200 ml/min, and each component was returned to the dog. Sodium oleate 3% (106 μEq/ml) in 0.082 M saline was infused into the plasma line with a mean ratio of 7.74 μEq (±1.27 SD)/ml of plasma flow per min. Control periods of 60 min preceded and followed each oleate infusion. During these periods, normal saline was infused at the same rate (milliliters per minute) used for sodium oleate. Anticoagulation was maintained by constant infusion of a concentrated heparin-saline solution so as to deliver 2000 U of heparin in a volume of 25 ml/hr.

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Pancreatic insulin secretion. Insulin levels in pancreaticoduodenal vein blood during oleate infusion were studied in two dogs. After a laparotomy under pentobarbital anesthesia, two polyethylene catheters (I.D. 1.2 mm) connected by a "T-fitting" were placed between the superior pancreaticoduodenal vein and the portal vein, respectively. Pancreatic blood flow, measured periodically by diverting blood flowing from the catheter into a graduated cylinder, did not vary by more than ±12%. The dogs were connected to the centrifuge by means of femoral artery–femoral vein shunts. All other conditions were the same as those for oleate infusions in the unanesthetized dogs.

Fat-feeding studies. Six unanesthetized dogs were connected to the IBM centrifuge as for oleate infusions. Arterial blood was separated into cells and plasma at the rate of 70 ml/min, and both fractions were returned to the dogs continuously. During a 6 hr control period, a glucose solution of 10 g/100 ml was infused at 1 ml/min; then each dog ingested 200 g of triolein[1] during 10–15 min, and the glucose infusion and centrifugation were continued for another 6 hr. Heparin was also administered continuously at the rate of 2000 U/hr throughout the study. The fat meal was omitted in eight additional dogs that served as 12-hr controls.

Chemical analyses. Blood was sampled from the arterial shunt line and pancreaticoduodenal vein at 10- to 15-min intervals and chilled immediately to 4°C to prevent lipolysis in vitro. After centrifugation at 4°C, the plasma was either analyzed at once or rapidly frozen for later determination. Measurement of plasma glucose and FFA were as noted in the previous paper (7).

Plasma immunoreactive insulin was assayed by a modification (8) of the double-antibody method of Morgan and Lazarow (9), using a crystalline pork insulin standard,[2] pork insulin-^{125}I,[3] guinea pig anti-pork insulin serum,[4] which reacted identically with pork and dog insulin, and rabbit anti-guinea pig γ-globulin serum.[4] Insulin values are the means of duplicate analyses, which agreed within 10%. In a representative assay of 51 pairs of duplicate samples (range: 2–116 μU/ml), the standard deviation between duplicates was 1.6 μU/ml (10). Consistency of assay results was maintained by performing all assays with aliquots of the same insulin standard and anti-insulin serum. The addition of sodium oleate to plasma in vitro did not affect the assay of either glucose or insulin.

Plasma ketone bodies were assayed by the method of Chernick (11). Differences between values were tested for significance by Student's *t* test.

RESULTS

Effect of sodium oleate infusion. When oleate was infused at high rates, plasma insulin rose to very high levels within 10 min after the start of the infusion. Blood glucose usually began to fall 10–20 min thereafter, and marked hypoglycemia developed (Table I). Lower elevations of FFA produced similar but smaller effects (Fig. 1). The fall in blood glucose, however, was not equally rapid in all experiments and was particularly slow in two dogs infused under pentobarbital anesthesia. As glucose levels fell during oleate infusion, plasma insulin often declined somewhat from initial peak levels. At the end of each FFA infusion (duration: 45–150 min), plasma FFA and insulin rapidly dropped to preinfusion levels, and plasma glucose rose toward normal.

Although in most experiments the maximum rise in insulin and fall in glucose occurred at 15 and 45 min after onset of the oleate infusion, respectively, some variation in the time of maximum response was apparent. Thus, for purposes of ready comparison of the magnitude of the responses in many experiments, the mean of five control values was compared with the mean of three successive samples obtained over the 30 min infusion period during which there was a maximum response for insulin and glucose, respectively. By these criteria, a significant rise in plasma insulin and fall in glucose occurred in almost all experiments (Table I).

Over the range of 1.0–2.0 μEq/ml, the magnitude of the rise in plasma insulin and fall in glucose correlated with the mean FFA level during infusion (Fig. 2). A minimum stimulatory level was not determined. Simultaneous infusion of glycerol[5] with oleate (1:3 molar ratio), in order to simulate endogenous lipolysis more closely, did not significantly alter the insulin and glucose responses.

Effect of oleate infusion on animals receiving a continuous glucose infusion. In four dogs infused with oleate (mean rate: 39 μEq/kg per min) while receiving a continuous glucose infusion (100 mg/min), the time course and magnitude of the changes in FFA, insulin, and glucose were similar to those found in dogs not given glucose. These responses also were not affected by simultaneous infusion of glycerol with oleate (Fig. 3). In one study, however (Experiment 16), oleate infusion at 80 μEq/kg per min produced a striking dissociation of the insulin and glucose responses. Mean plasma insulin rose to 725 μU/ml (SEM ±99), the highest level reached during any oleate infusion, but blood glucose declined very slowly.[6]

To determine whether the insulin and glucose responses were due to acid-base changes with FFA infusion, 3% sodium bicarbonate was infused at 140 μEq/kg per min into a dog receiving a continuous glucose infusion (100 mg/min) during centrifugation. In contrast to the effects of sodium oleate infusion, infusion of sodium bicarbonate produced a moderate fall, rather than a rise, in plasma insulin, and a slight fall in plasma glucose (14 mg/100 ml) after 45 min of infusion. The gradual fall in glucose seen in this experiment is a response that

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] Kindly supplied by Dr. Otto K. Behrens, Eli Lilly & Co. Research Laboratories, Indianapolis, Ind.
[3] ISO/Serve Division, Cambridge Nuclear Corp., Cambridge, Mass.
[4] Arnel Products Co., New York.
[5] Glycerin, A. C. S. grade, Allied Chemical Corp., Morristown, N. J.
[6] Tables containing the data of the individual animals have been deposited with The American Documentation Center.

TABLE I

*Summary of Effects of Oleate Infusion on Plasma FFA, Glucose, and Insulin**

Exp. No.	Dog	Infusion rate	Mean plasma FFA: Control	Mean plasma FFA: Infusion	Mean plasma FFA: Δ	Mean plasma glucose: Control	Mean plasma glucose: Infusion	Mean plasma glucose: Δ	P value		Mean plasma insulin: Control	Mean plasma insulin: Infusion	Mean plasma insulin: Δ	P value
		μEq/kg per min		*μEq/ml*			*mg/100 ml*					*μU/ml*		
High elevation of plasma FFA														
1	A	58.3	0.59 ±0.02	1.80 ±0.12	1.21 ±0.12	109 ±4	71 ±8	38 ±9	<0.01		7 ±1	31 ±5	24 ±5	<0.01
2	B	59.0	0.87 ±0.05	1.94 ±0.08	1.07 ±0.11	106 ±2	52 ±2	54 ±3	<0.01		6 ±1	72 ±55	66 ±56	NS
3	C	64.8	0.66 ±0.05	1.87 ±0.08	1.21 ±0.09	111 ±6	66 ±5	45 ±8	<0.01		8 ±3	64 ±3	56 ±4	<0.01
4	C	43.3	0.65 ±0.08	1.62 ±0.10	0.97 ±0.12	121 ±4	83 ±3	38 ±5	<0.01		26 ±5	115 ±25	89 ±25	<0.05
5	D	40.8‡	0.50 ±0.03	1.41 ±0.02	0.91 ±0.04	92 ±2	71 ±2	21 ±3	<0.01		6 ±1	49 ±13	43 ±13	<0.05
6	E	35.5§	0.36 ±0.03	1.57 ±0.03	1.21 ±0.04	123 ±2	95 ±1	28 ±3	<0.01		12 ±1	28 ±3	16 ±3	<0.01
7	D	29.5§	0.31 ±0.02	1.40 ±0.04	1.09 ±0.04	143 ±3	87 ±6	56 ±6	<0.01		14 ±3	43 ±5	29 ±6	<0.01
Mean ±SE		47.3 ±5.1	0.56 ±0.07	1.66 ±0.08	1.10 ±0.11	115 ±6	75 ±5	40 ±8			11 ±3	57 ±11	46 ±12	
Low elevation of plasma FFA														
8	F	37.2	0.56 ±0.03	1.05 ±0.07	0.49 ±0.07	106 ±3	90 ±6	16 ±7	NS		4 ±1	12 ±1	8 ±2	<0.01
9	D	36.0	0.41 ±0.01	0.99 ±0.05	0.58 ±0.05	131 ±3	99 ±0	32 ±3	<0.01		7 ±1	16 ±1	9 ±1	<0.01
10	F	24.8	0.57 ±0.04	0.98 ±0.03	0.41 ±0.05	106 ±1	98 ±1	8 ±1	<0.01		7 ±1	16 ±1	9 ±1	<0.01
11	G	19.4	0.55 ±0.05	1.07 ±0.05	0.52 ±0.07	114 ±2	103 ±2	11 ±3	<0.05		8 ±1	15 ±1	7 ±2	<0.01
Mean ±SE		29.4 ±4.3	0.52 ±0.04	1.02 ±0.02	0.50 ±0.04	114 ±6	97 ±3	17 ±6			7 ±1	15 ±1	8 ±1	
Simultaneous glucose infusion, *100 mg/min*														
12	H	32.1	0.34 ±0.01	1.33 ±0.04	0.99 ±0.04	128 ±3	103 ±1	25 ±4	<0.01		16 ±1	37 ±3	21 ±3	<0.01
13	I	45.7	0.34 ±0.04	2.02 ±0.07	1.68 ±0.09	139 ±2	65 ±2	74 ±3	<0.01		13 ±1	83 ±11	70 ±12	<0.01
14	J	34.8	0.37 ±0.03	1.42 ±0.03	1.05 ±0.05	153 ±3	99 ±4	54 ±5	<0.01		31 ±8	121 ±44	90 ±44	NS
15	K	43.6‡	0.45 ±0.04	1.67 ±0.04	1.22 ±0.05	135 ±5	93 ±1	42 ±5	<0.01		13 ±1	31 ±4	18 ±4	<0.01
16	L	80.0‡,‖	0.42 ±0.01	1.85 ±0.11	1.43 ±0.11	151 ±2	134 ±1	17 ±3	<0.01		39 ±2	725 ±99	686 ±99	<0.01
17	M	31.7¶	0.42 ±0.05	1.07 ±0.05	0.65 ±0.07	359 ±9	318 ±9	41 ±13	<0.05		76 ±1	156 ±22	80 ±22	<0.05
Mean ±SE		39.1 ±3.3	0.38 ±0.03	1.61 ±0.16	1.23 ±0.05	139 ±5	90 ±9	49 ±10			18 ±4	68 ±21	50 ±22	
Sodium Bicarbonate infusion, *140 μEq/kg per min*														
18	N	0	0.38 ±0.02	0.32 ±0.02	0.06 ±0.03	150 ±2	117 ±1	33 ±2	<0.01		40 ±4	21 ±3	−19 ±6	<0.05
Pancreatic vein study														
19	PV-1	19.6§	0.23 ±0.02	1.60 ±0.11	1.37 ±0.11	126 ±6	137 ±1	11 ±6	NS	FA	5 ±1	18 ±0	13 ±1	<0.01
										PV	147 ±32	399 ±47	252 ±57	<0.01
20	PV-2	36.5§	0.37 ±0.02	1.48 ±0.06	1.11 ±0.07	137 ±3	99 ±7	38 ±8	<0.01	FA	5 ±1	173 ±33	168 ±33	<0.01
										PV	55 ±6	3544 ±661	3489 ±661	<0.01

FA, femoral artery; PV, pancreaticoduodenal vein; NS, not significant; FFA, free fatty acids.

* Control values are means ±SEM of five separate determinations during the 1 hr of saline infusion which preceded each oleate infusion. FFA infusion values are means ±SEM of all FFA determinations during infusion, while glucose and insulin values are means ±SEM of three determinations during the 30 min infusion period of maximum response for glucose and insulin, respectively.

‡ Glycerol combined with oleate (1:3 molar ratio).

§ Dog anesthetized with pentobarbital (30 mg/kg).

‖ Extraordinary rate; values not included in group means.

¶ Glucose infusion at 500 mg/min; values not included in group means.

has often been observed in dogs receiving glucose infusions at a constant rate (12).

Effect on plasma ketones. Total ketone levels were measured in four dogs infused with oleate at high rates. Two of them received oleate during a continuous glucose infusion (K and L, Table I), and their ketone levels did not rise significantly, even when a plasma FFA level above 1.8 μEq/ml was maintained for over 75 min (experiment 16). In the other two dogs (B and C, Table I), ketone levels rose slightly during oleate infusion, but at the time of maximum insulin secretion did not exceed twice the control levels (0.27 mm/liter).[6] Moreover, throughout each oleate infusion studied, plasma ketones were always less than 20% of the high levels reported to stimulate insulin secretion in dogs (13, 14).

Effect of oleate infusion on pancreatic insulin secretion. In the two dogs studied, infusion of oleate produced a significant rise in pancreaticoduodenal vein insulin levels, and peripheral insulin levels mirrored those in the pancreatic effluent blood (Table II). At a high rate of oleate infusion, pancreaticoduodenal vein insulin levels increased to 80 times the control values (Fig. 4). In both experiments, plasma insulin declined somewhat from maximum levels before the end of the FFA infusion. Blood glucose declined less sharply in these experiments than during oleate infusion in unoperated dogs, perhaps due to metabolic consequences of surgery.

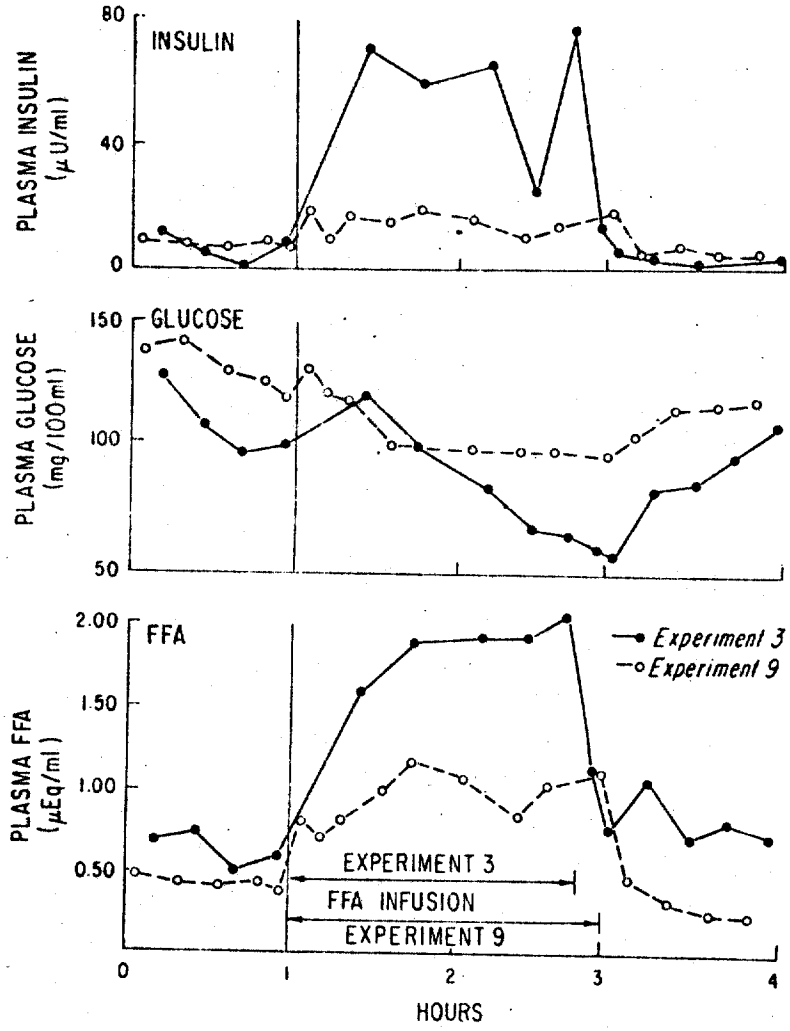

FIGURE 1 Comparison of the effect of sodium oleate infusion at high and low rates on plasma FFA, glucose, and insulin. After 1 hr of saline infusion, oleate was infused at 64.8 μEq/kg per min in experiment 3, and 36.0 μEq/kg per min in experiment 9.

Effect of fat feeding and heparin. After triolein ingestion and intravenous heparin, generally small increases in plasma insulin occurred, but they did not correlate well with the FFA levels reached, in contrast to the effects of oleate infusion. The rise in insulin was actually greater in the three dogs experiencing only a modest increase in FFA levels than in the three in which FFA rose to higher levels (above 1.50 μEq/ml) (Table III). Plasma glucose also rose slightly after the fat meal, but was not significantly greater than glucose levels in control dogs except in the 5th hr after fat ingestion (Fig. 5).

DISCUSSION

The results indicate that in dogs the acute elevation of plasma FFA levels by sodium oleate infusion is a potent stimulus of insulin secretion. Mean elevation of plasma FFA to 1.0 μEq/ml produced a 2-fold increase in peripheral plasma insulin concentration, and larger increases in FFA produced correspondingly higher insulin levels. The magnitude of the subsequent fall in blood glucose also correlated with the FFA level during infusion.

It is important to consider whether these responses could have resulted from any factor other than the elevated FFA level produced. As reported previously (7),

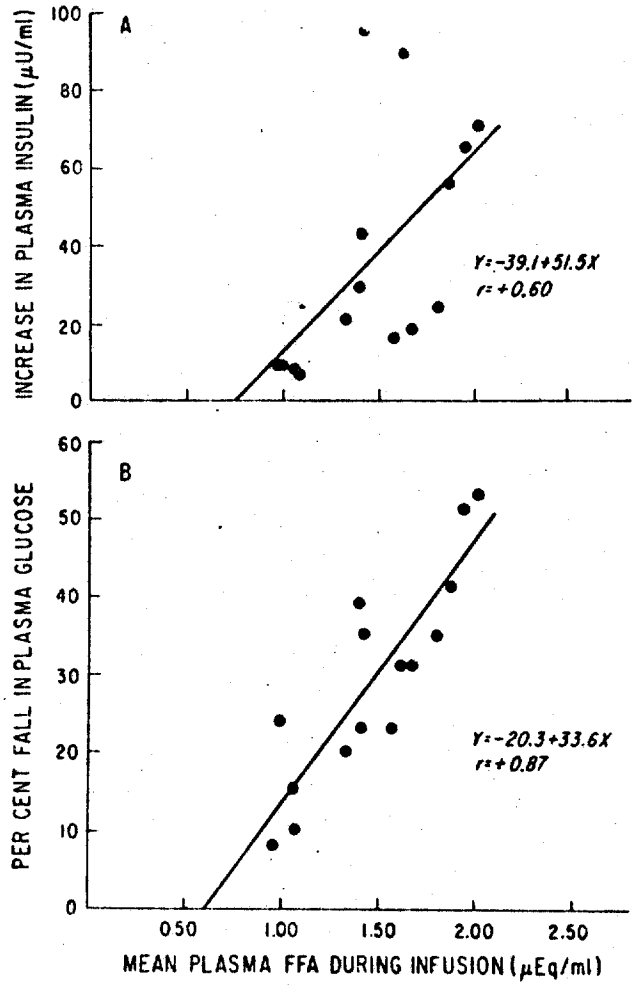

FIGURE 2 Regression analysis of the relation between mean plasma FFA during infusion and (A) increase in plasma insulin, and (B) percentage fall in plasma glucose, in experiments 1–15. Insulin and glucose responses were calculated from the differences between mean control levels and mean values during the 30 min infusion period of maximum response for insulin and glucose, respectively. In both cases, r was significant ($P < 0.02$ for A, $P < 0.01$ for B).

results of control studies of the metabolism of dogs subjected to continuous centrifugation make it unlikely that this process per se could account for the changes in insulin and glucose. In the present study, little change in plasma insulin or glucose occurred in the eight control dogs subjected to 12 hr of continuous centrifugation and heparinization, except for the gradual decline in blood glucose over several hours that is commonly observed during a constant glucose infusion (12). Moreover, no stimulation of insulin secretion was observed when the dogs were receiving heparin and saline during the 60 min control period that preceded and followed each oleate infusion. It seems unlikely that the entry of extracellular potassium into cells during the mild metabolic

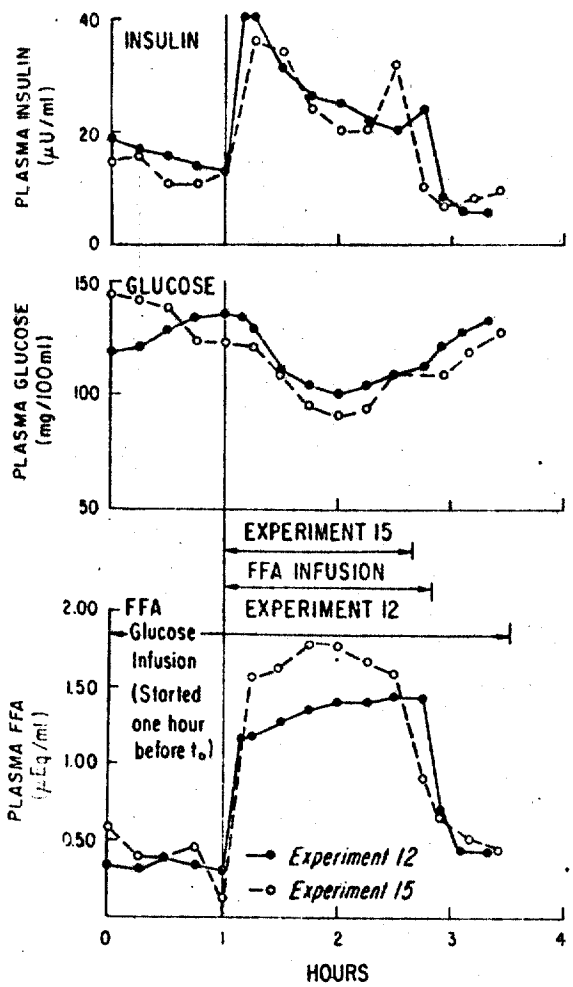

FIGURE 3 Comparison of the effect of sodium oleate infusion with and without glycerol on plasma FFA, glucose, and insulin during a constant glucose infusion (100 mg/min). Oleate was infused at 32.1 μEq/kg per min in experiment 12, and at 43.6 μEq/kg per min simultaneously with glycerol (14.5 μEq/kg per min) in experiment 15.

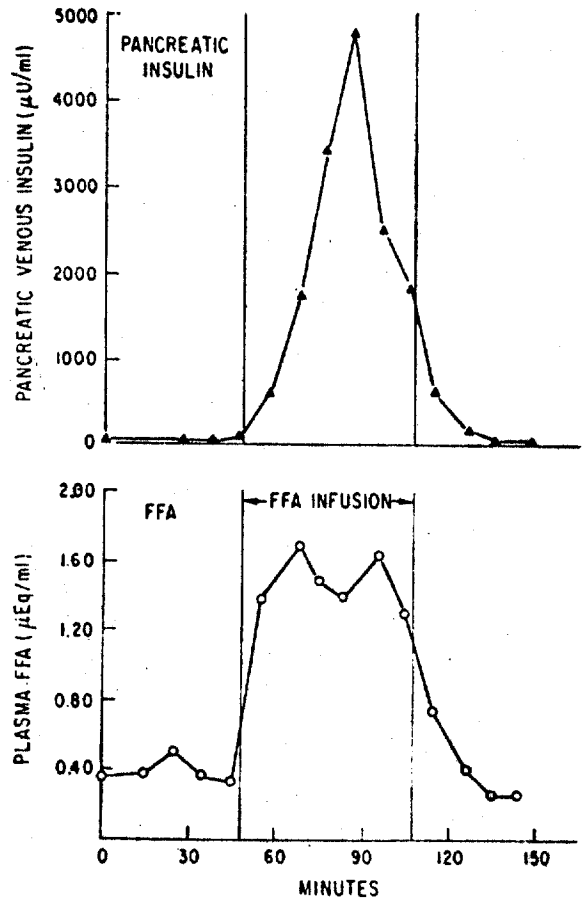

FIGURE 4 Effect of sodium oleate infusion (36.5 μEq/kg per min) on femoral arterial FFA and pancreaticoduodenal venous insulin.

alkalosis accompanying oleate infusion significantly affected insulin secretion, since no increase in plasma insulin was observed in the dog given a massive sodium bicarbonate infusion during centrifugation. It is also unlikely that sodium oleate evoked insulin release by stimulation of autonomic reflexes in the jugular vein or superior vena cava, since plasma insulin increased whether oleate was infused into the jugular vein or into the femoral vein.

Whether sodium oleate stimulates insulin release directly or whether the effect is indirect cannot be decided from these studies. It is unlikely that the insulin response was secondary to an increase in plasma ketone levels, since in most experiments they rose only slightly, if at all. Preliminary studies in our laboratory (15) have indicated that direct infusion of FFA into the pancreaticoduodenal artery of anesthetized dogs (at rates which did not raise systemic FFA) was in fact accompanied by a prompt and marked increase in pancreatic

TABLE II

Effects of Sodium Oleate Infusion on Plasma FFA, Glucose and Insulin, and Pancreaticoduodenal Venous Insulin

Exp. No.	Dog	Weight	Infusion rate	Measurement*	Preoleate control period, min				Sodium oleate infusion period, min									Postoleate control period, min		
					−45	−30	−15	0	10	20	30	40	50	55	60	70	100	10	25	50
		kg	*μEq/kg per min*																	
19	PV-1	15.9	18.6	FFA	0.22	0.23	0.19	0.23	1.28	1.36		1.89		1.70		1.88	1.83	0.80	0.32	0.28
				G	136	135	117	108	116	123		155		158		145	137	140	127	131
				IRI	2	4	4	6	3	8		11		16		15	18	15	9	9
				PV-IRI	254	186	75	101	93	140		359		493		344	253	117	197	132
20	PV-2	18.2	36.5	FFA	0.35	0.37	0.40	0.32	1.39	1.68	1.49	1.39	1.64		1.31			0.74	0.34	0.32
				G	143	128	136	144	140	139	139	144	155		136			129	106	116
				IRI	7	7	4	6	13	69	109	192	219		128			95	17	11
				PV-IRI	50		54	63	660	1733	3395	4756	2482		1814			640	144	55

* FFA, plasma FFA (μEq/ml); G, plasma glucose (mg/100 ml); IRI, plasma immunoreactive insulin (μU/ml); PV-IRI, pancreaticoduodenal venous insulin (μU/ml).

insulin levels. An apparently direct effect of FFA has also been reported recently by Malaisse and Malaisse-Lagae (16), who found that 0.5 mM palmitate stimulated insulin secretion in pieces of rat pancreas. On the other hand, Howell (cited by Montague and Taylor [17]), was not able to demonstrate an effect of palmitate on insulin secretion in rat pancreas slices, but the details of these experiments have yet to be published. The short-chain fatty acids butyrate and octanoate have been reported to stimulate insulin secretion both in vivo and in vitro (17–20).

Seyffert, Madison, Unger, and Barker (21) have reported that elevation of plasma FFA levels by intravenous infusion of a triglyceride emulsion and heparin stimulated insulin secretion in anesthetized dogs. They found that the elevation of plasma FFA from 390 to 1400 μEq/ml was accompanied by a rapid rise in plasma insulin from 45 to approximately 60 μU/ml in five dogs. Since the dogs were prepared with portacaval shunts, thus eliminating the appreciable hepatic uptake of insulin that normally occurs when pancreatic venous blood goes first to the liver (22, 23), the observed responses were probably maximal. Moreover, in contrast to the marked hypoglycemia seen at high FFA levels in the present experiments, after 90 min of triglyceride infusion Seyffert and Madison (4) observed (in 21 studies) a mean fall in blood sugar of only 14 mg/100 ml while plasma FFA rose from 0.54 to 1.83 μEq/ml. It seems likely that this more limited response was due in part to anesthesia, since in the present studies anesthesia was found to affect the time course of the glucose response to FFA infusion. Moreover, as discussed below, there may have been metabolic accompaniments of the indirect method used to raise FFA levels that also modulated the observed responses.

In the present studies, the individual contribution of hepatic glucose output and peripheral glucose utilization to the observed hypoglycemia cannot be assessed. It would appear that the magnitude and duration of the insulin response was such that both decreased hepatic glucose output and increased peripheral glucose utilization would occur (24). Nevertheless, it is quite possible that elevated FFA levels inhibited peripheral glucose utilization, but that hepatic glucose output was even more sharply reduced. Indeed, Seyffert and Madison calculated that the acute elevation of FFA by their method produced a 30% fall in peripheral glucose utilization and a 37% fall in hepatic glucose output. Moreover, an inhibitory effect of FFA on peripheral glucose utilization superimposed on the insulin effect in our studies could perhaps explain the dissociation of the insulin and glucose responses which occurred in some experiments. Thus the present studies are not incompatible with the glucose–fatty acid cycle hypothesis of Randle (25) who proposed that a reciprocal relationship exists between FFA and ketone metabolism on the one hand and the rate of peripheral glucose utilization on the other.

Although small increases in plasma insulin levels were noted after fat ingestion and intravenous heparin in some of the present studies, the increases did not correlate well with FFA level, as did the insulin response to oleate infusion. FFA levels, however, rose much more slowly in these fat-feeding experiments. It is also possible that in the hourly interval between samples, plasma insulin actually rose briefly to higher levels than those observed. Nevertheless, the present results appear similar to those in patients observed by Schalch and Kipnis (5) and Balasse and Ooms (6), who found that the acute elevation of FFA levels by injection of heparin after fat ingestion did not significantly affect basal insulin and glucose levels. Both groups, however, also found a significant decrease in the disappearance rate of glucose loads administered during FFA elevation.

TABLE III

Effects of Feeding Triolein on Plasma FFA, Glucose, and Insulin in Dogs Receiving Glucose Infusion (100 mg/min) during Continuous-Flow Centrifugation

Dog	Weight	Measurement	Hours after start of infusion 0	1	2	3	4	5	6*	7	8	9	10	11	12
	kg														
FF-21	19.5	FFA	1.51	0.73	0.37	0.43	0.41	0.38	0.37	0.37	0.58	0.63	0.83	0.89	0.74
		G	104	167	113	106	108	112	124	127	133	127	126	132	133
		IRI	7	5	8	7	8	9	7	6	7	9	11	19	17
FF-22	19.5	FFA	1.39	0.83	0.77	0.72	0.57	0.42	0.30	0.71	1.98	1.90	2.32	2.26	2.88
		G	93	168	202	197	174	151	123	107	121	121	140	153	149
		IRI	4	16	18	15	15	11	11	8	9	8	12	10	19
FF-23	18.3	FFA	0.98	0.50	0.42	0.39	0.39	0.36	0.43	0.74	0.80	0.98	0.99	0.95	1.05
		G	163	261	223	230	213	186	181	153	133	143	157	139	150
		IRI	12	49	35	39	45	46	24	43	45	54	73	55	56
FF-24	16.8	FFA	1.45	0.77	0.27	0.24	0.31	0.39	0.41	0.24	0.68	1.57	0.97	0.50	0.58
		G	113	173	193	170	163	188	188	141	122	125	136	138	126
		IRI	1	18	3	6	2	8	5	7	8	4	15	11	4
FF-25	20.0	FFA	1.70	0.94	0.58	0.78	0.57	0.62	0.62	0.53	0.93	0.70	0.54	0.51	0.59
		G	99	147	151	144	144	164	188	135	131	132	132	144	137
		IRI	1	9	12	9	9	5	6	14	9	11	17	17	13
FF-26	12.7	FFA	1.78	0.78	0.36	0.56	0.55	0.61	0.60	0.83	0.45	1.52	1.18	1.90	2.15
		G	99	194	170	147	151	171	152	147	158	144	137	133	133
		IRI	4	18	11	9	6	14	10	13	10	15	10	21	9
Mean ±SEM		FFA	1.47‡	0.76‡	0.46	0.52	0.47	0.46	0.46	0.57	0.90‡	1.22‡	1.14‡	1.17‡	1.33‡
		SEM	±0.12	±0.06	±0.07	±0.08	±0.05	±0.05	±0.05	±0.09	±0.23	±0.21	±0.25	±0.30	±0.39
		G	102	185	175	166	159	162	159	136	134	132	138	140‡	138
		SEM	±3	±16	±16	±18	±14	±11	±13	±7	±6	±4	±4	±3	±4
		IRI	5	19	15	14	14	16	11	15	15	17	23	22	20
		SEM	±2	±6	±5	±5	±6	±6	±3	±6	±6	±8	±10	±7	±8
Controls (n = 8) Mean ±SEM		FFA	0.90	0.52	0.38	0.35	0.38	0.35	0.36	0.39	0.37	0.37	0.35	0.35	0.34
		SEM	±0.11	±0.05	±0.03	±0.04	±0.05	±0.04	±0.04	±0.05	±0.04	±0.04	±0.03	±0.03	±0.02
		G	104	158	159	144	141	143	141	132	134	136	131	121	129
		SEM	±2	±13	±11	±10	±9	±8	±10	±7	±5	±7	±4	±6	±3
		IRI	6	15	16	15	13	14	15	13	13	15	14	12	13
		SEM	±2	±2	±3	±3	±2	±2	±2	±2	±2	±3	±3	±2	±3

FFA, plasma FFA (μEq/ml); G, glucose (mg/100 ml); IRI, immunoreactive insulin (μU/ml).
* 200 g triolein fed at start of 6th hr.
‡ Significantly greater ($P < 0.05$) than mean control value in corresponding period.

It is necessary to ask why the insulin and glucose responses apparently differed when FFA levels were raised by direct infusion as opposed to elevation by feeding fat in the presence of heparin. In the process of absorption of a fat meal, there is opportunity for a selective intestinal hormonal response (26). Under these conditions, there might, for example, be release of a hormone from the gut that inhibits insulin secretion, directly or indirectly. Another possibility is that there are metabolic accompaniments of the process of chylomicron removal beyond increasing intravascular FFA levels. For example, Ontko and Randle (27) have shown that chylomicrons inhibit glucose utilization in the perfused rat heart independent of any increase in FFA levels in the perfusate. One might speculate that chylomicrons have a direct metabolic effect on islet tissue tending to decrease insulin secretion. Another significant difference between the methods for FFA elevation is that when FFA levels were raised by oleate infusion there was no associated increase in lower glycerides and glycerol such as accompanies the action of lipoprotein lipase on chylomicrons. However, we observed no change in response during simultaneous infusion of glycerol with oleate. Finally, it is perhaps relevant that during infusion the rise in FFA was due primarily to an increment in oleate, instead of a mixture of fatty acids. However, we have recently found that infusion of linoleate also stimulated insulin secretion (15), and, as previously mentioned, palmitate has been found to stimulate insulin secretion in vitro (16). Moreover, the elevated FFA levels in the studies of Seyffert and Madison (4) presumably were due to increased levels of several fatty acids, reflecting the composition of the cotton seed oil emulsion which was infused.

Whether long-chain fatty acids play a significant role in the physiological regulation of insulin secretion remains to be established. During starvation, both plasma glucose and insulin levels have been reported to fall in

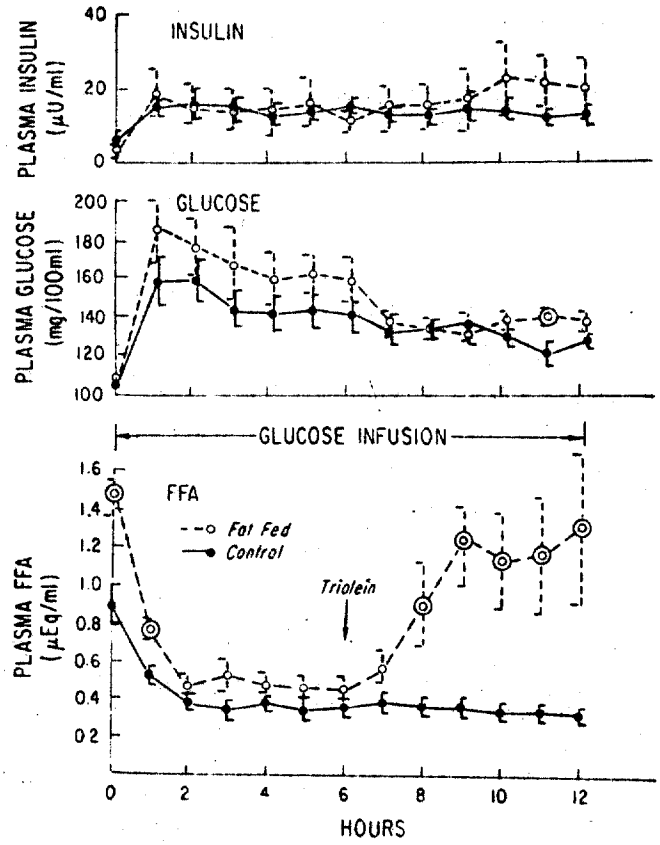

FIGURE 5 Effect of triolein feeding (200 g) on mean (±SEM) plasma FFA, glucose, and insulin in six dogs receiving a constant glucose infusion (100 mg/min) during 12 hr of continuous-flow centrifugation. Doubly circled values differed significantly ($P < 0.05$) from corresponding mean values of eight control dogs not fed triolein.

dogs and man (28). Thus, moderately elevated FFA levels do not appear to have a marked effect on insulin secretion in the metabolic setting of the starved organism. However, without the stimulus of FFA, insulin levels in starvation might be even lower than observed (4). Furthermore, a stimulatory effect of FFA on insulin could serve as a feedback control on the rate of lipolysis in adipose tissue (1). In addition, this FFA effect might modulate the inhibition of insulin secretion by epinephrine (29), one of the major physiological mediators of lipolysis.

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Platelet Adhesiveness and Euglobulin Lysis Time in Dogs Given Repeated Infusion of Sodium Palmitate

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Previous "in vitro" research suggested that serum lipids are not themselves inhibitors of fibrinolysis (5), and do not increase platelet adhesiveness (11). It was however demonstrated that sodium salts of longchain saturated fatty acids added "in vitro" to plasma, in physiological concentration, accelerate coagulation (16) and thrombus formation (3) and also produce an aggregation of washed platelets, (6).

Since low density lipoproteins may be produced in the liver from mobilized fatty acids (2), one might be tempted to suppose that an exaggerated mobilization of free fatty acids could be a common cause for both hyperlipemia and hypercoagulability in atherosclerotic patients.

Inhibition of fibrinolysis by free fatty acids was also reported (22) but in a concentration exceeding even pathological levels. However, this does not exclude a possible correlation "in vivo" between increased mobilization of lipids and inhibition of fibrinolysis. We have therefore decided to study the effect of repeated infusion of free fatty acids on platelet adhesiveness and fibrinolytic activity in dogs.

Material and Method

Rapid infusions of sodium salts of long chain saturated fatty acids are known to produce massive thrombosis and death (4, 7). On the contrary slow infusions of sodium palmitate were well tolerated (22). We have tried to administrate sodium palmitate in an amount and at a rate that should avoid acute death of the infused dogs, but in the same time to produce evident humoral changes. Sodium palmitate emulsion 3.5 m Mol, 7 ml/kg was given intravenously, during 7 min, in 16 mongrel dogs, 12 males and 4 females, weighing 9–20 kg.

A strikingly different reactivity was noted:

a) 6 animals died within 6–160 min after the end of the first infusion with sudden asphyxia and circulatory insufficiency. In 2 animals that died during infusion an emboly with precipitated soaps was suspected. These 2 animals were not included in the calculations.

b) 10 dogs, survived the first infusion and were injected again after 3 and 24 hrs and then daily until they died. In 2 animals the infusions were stopped for 48 hrs and then given again daily slow infusions. Four animals died spontaneously after respectively 5, 14, 26 and 30 days from the first infusion; the other 6 dogs were killed after 15–20 days from the first infusion, by rapid injection of sodium palmitate (10–15 ml/kg during 3–4 min). It is noteworthy that animals that survived the first infusion, and were given daily injections, proved to be subsequently resistent to doses of sodium palmitate that would have produced acute death in new animals. Therefore lethal rapid perfusion had to be given after an interruption of the perfusions for 48 hrs.

Before the first infusion and then 15 min, 3 hrs and 24 hrs later, blood (9 ml) was harvested with a siliconized canula from saphenous vein or jugular vein directly into a silicone coated, marked 10 ml test-tube containing 1 ml trisodium citrate 3.8%. Blood for examinations was taken subsequently every 48 hrs.

Platelets were counted in platelet rich plasma diluted 5000 times with acid ammonium oxalate 1%. A celoscope "TUR" ZG_1 with a 80 μ orifice size aperture tube, a low threshold setting of 4, non active high threshold discriminator, and an aperture current of 4 was used. Readings were done at room temperature within 30 min after the dilution of platelet rich plasma. Previous assays performed in 49 samples of platelet rich plasma demonstrated a good correspondence between celoscope countings and visual countings carried out according to Brecher and Cronkite. When using NaCl 0.9% in counting solutions lower values were obtained for machine platelet counts, owing probably to increased conductibility.

Platelet adhesiveness was measured in citrated platelet rich plasma, using the rotating bulb method of Wright (28). Results were expressed in percent of the prerotation value.

Plasma fibrinogen was assayed in platelet poor plasma according to Ratnoff and Menzie (17).

Fibrinolytic activity was appreciated as euglobulin lysis time using a modification of von Kaulla's method (24); 1 ml citrated platelet poor plasma was diluted with 20 ml ice cold acetic acid 0.015%. After centrifugation for 10 min at 3000 r.p.m. the precipitated euglobulins were dissolved in 1 ml barbiturate buffer (Owren) mixed before use with an equal amount of saline; 0.4 ml of the dissolved euglobulins were transferred into test-tubes at 37° C and clotted with thrombin. Euglobulin lysis time was measured also in progessive dilutions (1/1, 1/2, 1/4) of the euglobulin solution (0.2 ml) added with 0.2 ml bovine fibrinogen 200 mg% (Kabi Grade B_1) and coagulated with 0.1 ml thrombin (Topostasin Roche) containing 10 NIH/ml.

Free fatty acids were measured photocolorimetrically according to Novak, (13).

Table 1. Platelet Counts, Platelet Adhesiveness and Euglobulin Lysis Time in Dogs that Survived the First Infusion of Fatty Acids (1–10) and in Dogs that Died Soon after Infusion (11–14). I = initial values; II = 15 min after the beginning of the infusion; III = 3 hrs later. IV = after daily perfusion for 5 consecutive days. Blood harvested 24 hrs after the fifth perfusion.

Animal	Sex	Platelet count × 10^3				Adhesiveness %				Euglobulin lysis time/min			
		I	II	III	IV	I	II	III	IV	I	II	III	IV
1	M	301	236	342	–	77	12	0	–	70	60	120	–
2	F	479	246	433	380	84	10	0	12	59	13	110	420
3	M	330	176	240	–	80	30	31	–	40	20	110	–
4	F	715	571	779	530	72	13	19	23	60	60	300	240
5	M	238	201	300	260	22	15	13	16	60	33	140	180
6	M	126[1])	56	100	–	71	44	41	–	70	26	80	–
7	F	490	251	456	342	70	41	2	30	50	30	45	60
8	F	765	488	595	420	31	25	19	20	60	60	60	120
9	M	585	200	732	560	55	30	39	19	45	20	50	80
10	M	460	280	470	–	82	20	14	–	90	15	420	–
Mean		489	294	483	415	64	24	21	20	60	34	144	183
Standard error		±58	±56	±62	–	±6	±4	±4	–	±5	±7	±42	–
11	M	500	160	–	–	70	23	–	–	60	30	–	–
12	M	652	106	–	–	41	24	–	–	70	30	–	–
13	M	336	107	–	–	55	20	–	–	60	20	–	–
14	M	331	80	–	–	48	16	–	–	30	10	–	–
Mean		471	113	–	–	54	21	–	–	55	23	–	–
Standard error		±77	±17			±6	±1,8			±8	±5		

1) Animal No. 6 presented low initial platelet count. The thrombopenia was aggravated during infusions and reached values of 34,000, on the eighth day since the first infusion. This animal was not included in statistical calculations.

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Histologic Investigations in samples stained with haematoxylineosin and PAS were performed in 4 dogs that died during the first injection and in 9 animals that survived and received repeated infusions. The first group of 4 dogs was used as control for the more complex histological changes developing in dogs given repeated infusions.

Results

Platelets. As shown in Table 1, 15 minutes after the infusion a decrease of platelet counts was noted in every animal. The decrease was more accentuated in animals that died soon after the infusion (76% of initial values). In animals that survived, the decrease was 40%. Three hrs later, platelet counts were similar to those found before infusion. After repeated daily infusions, platelets were found to vary within normal limits when counted 24 hrs after the infusion. In only 1 dog with initial platelet values of 126.000, a thrombopenia of 34.000 was noted after 8 days.

Platelet Adhesiviness was found to decrease significantly ($p < 0.01$) already 15 min after the infusion. No difference could be established, with respect to adhesiveness between animals that died and animals that survived the first infusion. In the later group, platelet stickiness remained low also after 3 and 24 hrs. When infusions were discontinued for 48–72 hrs, a tendency to normalization of platelet adhesiveness was noted (see Fig. 4).

Plasma fibrinogen was not found to decrease after the first infusion. In most animals the slight changes could be accounted as an effect of dilution. In only 1 dog that died during infusion, fibrinogen values decreased from 310 mg% to 55 mg%. On the contrary in all animals that survived more than 7 days, a progressive increase of plasma fibrinogen was noted (Fig. 1). The great dispersion of initial values, makes it difficult to appreciate statistically the results.

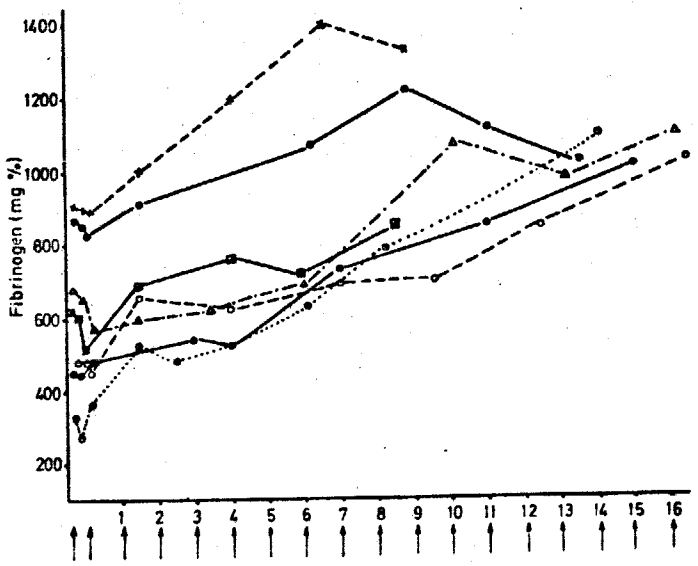

Fig. 1. Increase of plasma fibrinogen in dogs that survived more than 7 days after the first infusion. On the abscisa: time in days; on the ordinate: plasma fibrinogen (mg%). Arrows represent infusions of sodium palmitate emulsion.

Euglobulin lysis time became accelerated at the end of the infusion ($0.01 < p < 0.02$) This transient acceleration was replaced by a delayed lysis time 3 hrs later (see Table 1) However, the difference was not statistically significant when compared with initial

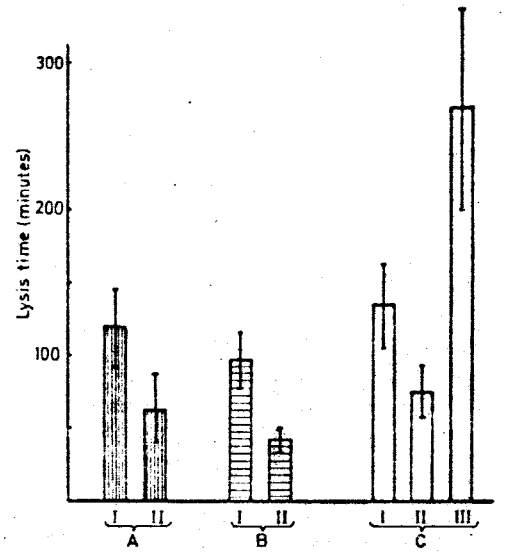

Fig. 2. Lysis time of euglobulins added bovine fibrinogen. a) Mean values obtained in all the 14 animals; b) mean values obtained in the 4 animals that died soon after the first infusion. c) Mean values obtained in 10 animals that survived the first infusion; I = before the infusion; II = 15 min after the beginning of the infusion; III = 3 hrs later. On the ordinate lysis time in min, Vertical lines represent standard deviation of the mean.

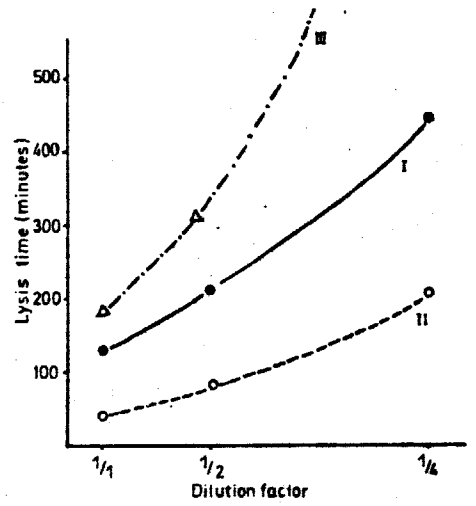

Fig. 3. Effect of the dilution of the euglobulins upon their lysis time. Bovine fibrinogen was added to the diluted euglobulins before clotting. On the abscissa: dilution factor; on the ordinate: lysis time in min. I = before infusion; II = 15 min after infusion; III = 3 hrs later. Illustrative samples

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lysis time. The delay of fibrinolysis in samples harvested 3 hrs after infusion, was more evident when euglobulins were added bovine fibrinogen ($p<0.02$). Dilution of euglobulins makes the difference still more striking (Fig. 3).

Depression of fibrinolytic activity persisted at least 24 hrs after the infusion. When infusions were discontinued for 48–72 hrs a tendency to normalization of fibrinolytic activity was noted.

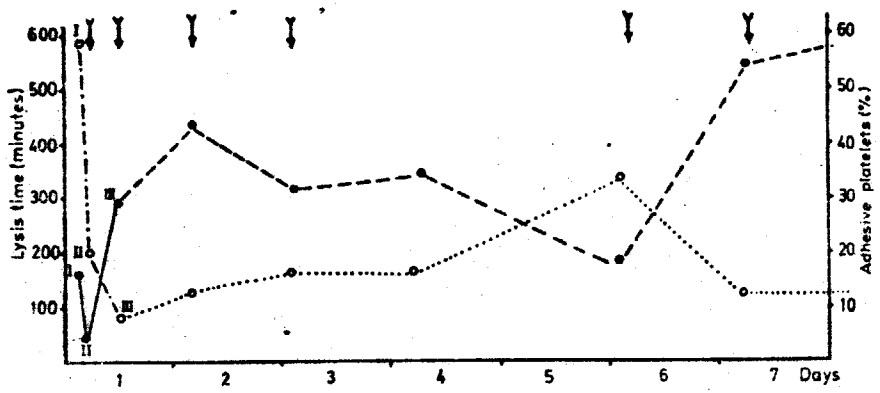

Fig. 4. Euglobulin lysis time in min (.—.) and platelet adhesiveness in percentage (o...o) in a dog given repeated infusion of sodium palmitate. Illustrative experiment; on the abscissa: time in days, since the first infusion. The first 3 determinations represent values obtained before the infusion (I), 15 min (II), and 3 hrs (III) after the first infusion. Euglobulins were added bovine fibrinogen before clotting.

Concentration of free fatty acids in serum did not change 15 min after the intravenous injection of sodium palmitate. Values obtained in samples of blood harvested from different vascular territories during infusion of fatty acids in 2 animals anesthetized with chloralose, are presented in Fig. 5.

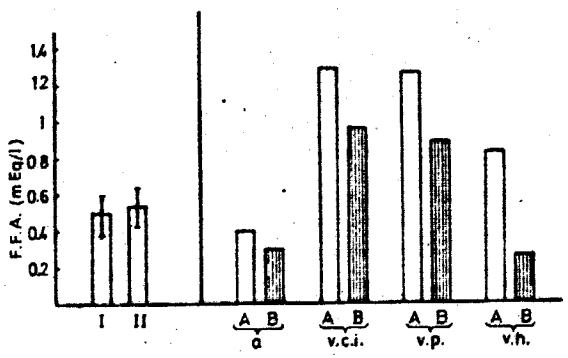

Fig. 5. Concentration of unesterified fatty acids before (I) and 15 min after the infusion of sodium palmitate (II). The second part of the figure represents concentration of unesterified fatty acids in blood harvested in 2 dogs (A and B) from different vascular territories: a = saphenous vein before infusion; v.c.i. = inferior vena cava during infusion; v.p. = pulmonary vein during the infusion; v.h. = hepatic vein during infusion.

Histological investigation emphasized the deposition of PAS positive material, consistent with fibrin or agglutinated platelets in the renal glomerulus and pulmonary capillaries. Acute changes were characterized by capillary fragility or erythropermeability. In 7 animals that survived the first infusion, more complex histological aspects were seen, with proliferation of endothelial cells in various degrees, suggesting an obliterating thrombangiitis. No aspects of thrombangiitis was noted in animals that died during the first infusion.

Fig. 6. Lung of a dog that died soon after infusion. Blood vessel with surrounding erythrocytes; hematoxylin and eosin stain (approx. × 140).

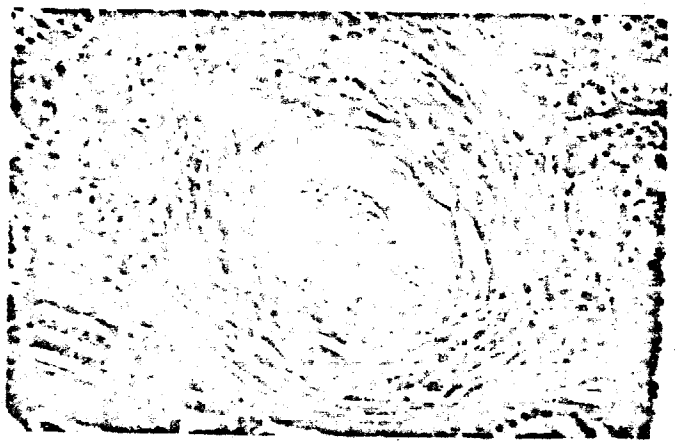

Fig. 7. Kidney of a dog that died 14 days after the first infusion. Obliterating thrombangiitis. Hematoxylin and eosin stain (approx. × 140).

Discussion of the Results

Decrease of blood platelets after intravenous infusions of long chain saturated fatty acids was previously reported by Zbinden (28) and by Connor et al. (4).

The greater decrease of platelet counts in animals that died soon after the first infusion might point to a possible role played by platelets in the determination of the susceptibility to the noxious effect of sodium palmitate but does not prove that death was produced by thrombosis.

We cannot state whether deficient stickiness of the remaining platelets and of those newly produced and/or mobilized is due to an impairment of platelets or to a consumption of some plasma factor necessary for adhesiveness. One might suppose however, that increased resistence to subsequent infusions, of the dogs that survived the first administration of fatty acids, could be conditioned also by this acquired impairment of platelet functions. In contrast to fatty acids resulting from endogenous metabolism, the infused sodium palmitate was not fixed on serum albumin. The effect on blood platelets should be rather explained by physico-chemical properties of sodium palmitate emulsion than by some metabolic action of fatty acids, especially as blood level of fatty acids was not changed 15 min after the infusion.

Decrease of platelet count and impaired platelet functions such as decrease of platelet factors 1–3 (18), deperdition of platelet ATP and deficient clot retraction (25) were also reported during the generalized Shwartzman phenomenon.

The increased fibrinogen level noted in animals that survived more than 7 days after the first infusion, could be correlated with the stimulating effect of fatty acids upon fibrinogen synthesis as reported by Pilgeram and Pickart (15) in an "in vitro" system based upon human liver biopsy samples. Hyperfibrinogenemia might be also interpreted as a consequence of necrotic phenomena produced by obliterating microthrombi.

It has been shown that activation of fibrinolysis diminish the deposition of fibrin produced by intravascular coagulation (19) while inhibition of fibrinolysis enhances the deposition of fibrin (1, 9). However, a compensating activation of fibrinolysis in peripheral blood was not emphasized during the generalized Shwartzman phenomenon. A delayed euglobulin lysis time was even noted (8, 10), a fact that does not exclude a local fibrinolysis at the level of fibrin depositions (24).

In our experiments we have distinguished a transient activation of fibrinolysis in peripheral blood followed by a delayed euglobulin lysis time. This seems to be a consequence of the exhaustion of blood activator that is probably adsorbed on fibrin.

It seems odd that the transient activation of fibrinolysis was greater in animals that died shortly after the first infusion. It probably represents an unspecific reaction to stress and coincides with an increased production of polypeptides acting on vascular permeability and circulation. Therefore a distinction should be made between such acute phenomena and subsequent evolution of microthrombi. One might suggest however, that proliferation of endothelia found in dogs given repeated infusions could be interpreted as a response to increased solicitation of the fibrinolytic system, as endothelia are known to produce activators of fibrinolysis (26). Possible implications of pluricausal thrombohaemorrhagic phenomena in clinical and experimental pathology are to be found in a recent monograph written by H. Selye (20). It is difficult to compare the results obtained in dogs infused sodium palmitate with changes in platelet adhesiveness and fibrinolytic insufficiency noted in clinical atherosclerosis (11, 12, 19). It seems however, that the relations between mobilization and metabolism of lipids and changes of coagulability are much more complex and probably imply the stimulation of the synthesis of some proteic factors involved in haemostasis.

Summary

The effect of infusions with sodium palmitate 3.5 m Mol/l on platelet adhesiveness and euglobulin lysis time was studied in 16 dogs. A decrease of platelet adhesiveness was noted 15 min after infusion. Decreased platelet adhesiveness persisted 3 and 24 hrs later, although platelet counts that were diminished 15 min after the infusion, returned to the initial level. Fibrinogen levels were not found to decrease during infusions of fatty acids. A progressive increase of plasma fibrinogen was even noted in animals that survived more than 7 days after the first infusion. Following a transient acceleration of euglobulin lysis time, a delayed fibrinolysis was noted, probably as a result of the exhaustion of blood activator.

Fibrin depositions were emphasized in histological sections. In some animals aspects of obliterative thromboangiitis were noted. However bilateral cortical necrosis was found in only 1 dog.

It seems that correlations between increased lipid mobilization and changes of coagulability and fibrinolysis in atherosclerosis are much more complex.

Résumé

On a étudié chez 16 chiens, l'effet des infusions de palmitaté de sodium, 3,5 m Mol/l sur l'adhésivité plaquetaire et sur le temps de lyse des euglobulines.

On a constaté une diminution de l'adhésivité des plaquettes 15 min après l'infusion. Cette baisse de l'adhésivité persistait trois heures et 24 h après l'infusion, quoique le nombre des plaquettes, riduit 15 min après l'infusion, soit revenu au niveau initial. Le taux du fibrinogène plasmatique ne diminue pas pendant les infusions. On a constaté même un accroissement progressif de la fibrinogénémie chez les animaux qui ont survécu plus de 7 jours après la premierè infusion.

Suivant une activation passagère de la fibrinolyse, on a constaté un prolongement du temps de lyse des euglobulines, que l'on cherche à expliquer par un épuisement de l'activateur sanguin.

Dans les coupes histologiques on a démontré des dépots de fibrine. Chez quelques animaux on a observé des aspects de thrombangéite oblitérante. Tout de même la nécrose corticale bilatérale des reins n'est évidente que pour un seul chien.

Il semble que les corrélations entre l'accroissement de la mobilisation des lipides et les modifications de la coagulabilité et de la fibrinolyse dans l'athérosclérose sont beaucoup plus complexes.

Zusammenfassung

Die Autoren haben die Wirkung von Natriumpalmitatinfusionen (3,5 mmol/l) auf die Thrombozytenadhäsivität und auf die Euglobulinlysiszeit an 16 Hunden untersucht. 15 Min nach der Infusion kam es zu einer Verminderung der Thrombozytenadhäsivität, die 3–24 Std. andauerte, wobei die Zahl der Blutplättchen 15 Min. nach der Infusion abfiel und später zu den Anfangswerten zurückkehrte. Die Fibrinogenwerte sanken nicht während der Infusion. Bei Tieren, die die erste Infusion mehr als 7 Tage überlebten, wurde ein Anstieg des Fibrinogens beobachtet. Auf eine vorübergehende Beschleunigung folgte eine Verzögerung der Fibrinolyse, wahrscheinlich als Folge der Erschöpfung des Blutaktivators.

Histologisch wurden Fibrinabscheidungen nachgewiesen. Bei mehreren Tieren kam es zu einer Thrombangiitis obliterans. Beidseitige Nekrose der Nebennierenrinde wurde nur in einem Fall beobachtet.

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Es ist anzunehmen, daß die Zusammenhänge zwischen erhöhter Fettmobilisierung einerseits und Blutgerinnung und Fibrinolyse andererseits bei Atherosklerose viel komplexer sind.

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Trans, t Across the Intestine, W.L. Burland and P.D. Samuel 20 (Eds.), Chap. 20, 1972

A REVIEW OF FAT TRANSPORT

A. M. DAWSON

DIETARY triglyceride is absorbed through the mucosal cell predominantly as the hydrolytic products, beta monoglyceride and free fatty acid. Bile facilitates their entry but is not obligatory as it seems to be for cholesterol, other sterols and fat soluble vitamins. Once inside the cell these fragments are predominantly reconstituted into tryglycerides and thence partially coated with a mixture of lipoprotein, phospholipid and cholesterol ester before they pass through the sides of the cell into the lymphatic spaces as chylomicrons and, as recently described, very low density lipoprotein (Dawson, 1967; Johnston, 1968; Senior, 1964).

Although bile salts are almost obligatory for the absorption of cholesterol and other sterols, up to 50 per cent of triglycerides may be absorbed in their absence (Dawson, 1967). It was initially assumed that decreased absorption was due to the lack of formation of micelles which facilitate the transfer of glyceride lipolytic products from the oil phase or luminal debris to the spaces between the microvilli and that some other less effective dispersing agent, such as a peptide, may be involved. Recently, using animals with lymph fistulae and lymph transport of absorbed fatty acid as an index of total absorption, it has been suggested that simple emulsification without micelle formation may be as efficient as when micelles are present (Simmonds *et al.*, 1968). On the other hand, more recent experiments have confirmed the importance of micelles. The absorption of oleic acid from segments of tied off rat intestine were studied and it was found that the absorption rate of oleic acid from a micellar solution was greater than from a solution emulsified with a lower concentration of bile salts (Fig. 1).

It seems unlikely that the suggestion of Morgan and Borgstrom (1969), that in the absence of bile, monoglyceride is hydrolysed in the lumen to free fatty acid before it can be absorbed, is true since it has recently been shown in our laboratory that mono-ether can be absorbed nearly as well as fatty acid in the absence of bile. The ether linkage is resistant to pancreatic hydrolysis. It seems likely that the monoglyceride hydrolysis takes place within the mucosal cell.

The uptake of monoglyceride, fatty acid and other lipids from micelles must account for the different rates of absorption of different lipids and could possibly occur in three ways:

(1) from the molecular phase, that is the phase through which the micelles

equilibrate with other micelles and the luminal oil phase or sediment (Simmonds *et al.*, 1967);

(2) the whole micelle in contact with the brush border disintegrates, allowing preferential uptake of different moieties of the micelle; and

(3) absorption of the whole micelle with subsequent rejection of different moieties at different rates (Feldman & Borgstrom, 1966; Gordon & Kern, 1968).

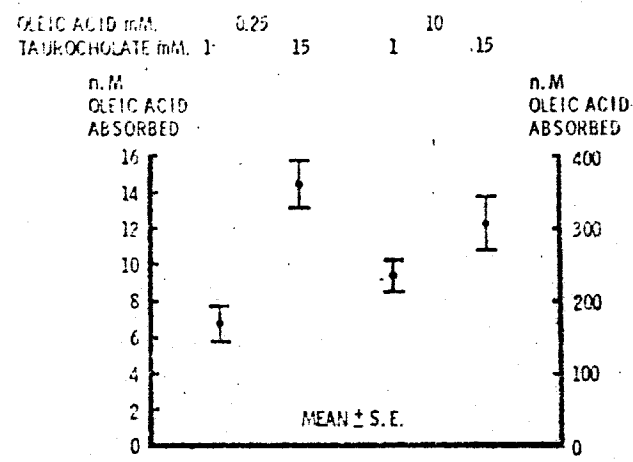

FIG. 1. Effect of bile salt micelle formation on absorption of oleic acid from rat jejunal segments *in vitro* (pH 6·5). Micelles are not formed by 1 mM taurocholate (Webb & Dawson, unpublished observation).

The third seems extremely unlikely but the initial argument that bile salts are not absorbed, is not necessarily valid for such studies, it excludes translocation across the cell and absorption into the circulation but not into the cell, and subsequent rejection into the lumen. The evidence for contact between the micelle and the brush border membrane or the intermediate molecular phase is equivocal. Presumably this latter mechanism would be necessary, at least for cholesterol and other very insoluble lipids, as they are virtually insoluble in an aqueous phase. The problem of using an *in vitro* system to study uptake of lipids is one of the reasons why this question is unresolved. Although everted sacs and other *in vitro* preparations of intestine can be of great help in studying transport systems of water soluble material, there is some doubt about their validity for lipids. It was shown by Sylven (1970) that everted segments of rat small intestine take up sitosterol and cholesterol from bile salt micellar solutions of these compounds to an equal degree (Fig. 2), thus confirming the observations of Feldman and Borgstrom (1966). However, when he used an *in vivo* system, i.e. tied off loops

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of rat small intestine, he found there was a selective uptake of cholesterol as compared with sitosterol. This *in vivo* selectivity, which is reminiscent of the total absorption of the two compounds, could be abolished by hypothermia but the selectivity returned once the animal had been warmed. It could also be abolished by rendering a segment ischaemic or by poisoning with cyanide. Thus there seems to be some property of the membrane which is energy dependent and is lost in the *in vitro* system. Different results were also obtained from *in vivo* and *in vitro* systems in studies of oleic acid uptake by rat small

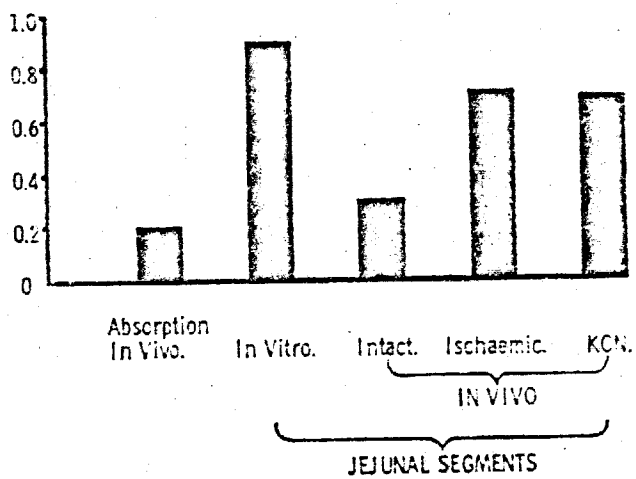

FIG. 2. Ratio, sitosterol/cholesterol uptake by the rat jejunum from bile salt micellar solution. Initial ratio unity (after Sylven, 1970).

intestine (Webb *et al.*, 1969). *In vitro* observations suggested that the molecular phase is important in the uptake of oleic acid from micelles for there was a predictable change in uptake from micellar solutions of oleic acid by an *in vitro* segment of rat jejunal mucosa. There was less uptake of the fatty acid with an increase in pH or bile salt concentration of the micellar solution. The effect of pH was not seen with the non-ionized oleyl alcohol and similar differences were not observed when *in vitro* systems were used.

Once the fatty acid or monoglyceride has come into contact with the brush border it probably diffuses through the membrane (Hogben, 1966). It would, in fact, be extremely difficult to demonstrate a so-called active transport mechanism for there is a rapid conversion of fatty acid to glyceride which in itself could presumably maintain a concentration gradient from the outside to the inside of the cell. The resynthesis of the fatty acid and monoglyceride into triglyceride is thought to take place in the endoplasmic reticulum. This theory is based on two pieces of evidence. On the one hand, the endoplasmic

reticulum is rich in enzymes necessary for this resynthesis to take place (Brindley & Hubscher, 1965; Senior & Isselbacher, 1962). On the other hand, electron microscope studies of the absorptive cell during fat absorption show fat between the microvilli but no stainable fat until deep within the cell, beyond the terminal web (Strauss, 1968). It would seem most likely that the fatty acids and the monoglycerides are attached to protein and thence transported to the endoplasmic reticulum in a finely dispersed form, although a specific carrier protein has not yet been identified. Isolated brush borders have been shown to be capable of activating and esterifying long chain fatty acids but the main moiety formed is phospholipid, rather than glyceride (Forstner *et al.*, 1968a), and is different from the microsomal system. Furthermore, on feeding rats with diets containing different fatty acids the brush

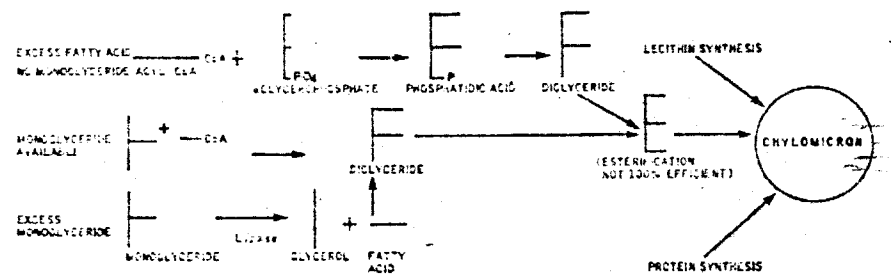

FIG. 3. Scheme for chylomicron formation in the intestinal mucosa.

border fatty acid profile partially mirrors the dietary lipid (Forstner *et al.*, 1968b). Although theoretically this potential could be used for the resynthesis of dietary fat it seems more likely that it is a mechanism for replenishing the brush border membrane.

There are two pathways for the resynthesis of triglyceride within the mucosal cell (Fig. 3). These have been reviewed by Johnston (1968). One, which is peculiar to the intestinal mucosa, involves the direct acylation of the absorbed monoglyceride with activated free fatty acid, so conserving energy. The other is the α-glycerophosphate pathway which is ubiquitous to most cell systems. This involves the acylation of α-glycerophosphate with acyl-CoA derivatives to form phosphatidic acid, dephosphorylation of the phosphatidic acid to form diglyceride and further acylation to form triglyceride. One species variation which has been noted is that in the sheep resynthesis takes place by the α-glycerophosphate pathway and no monoglyceride pathway exists (Bickerstaffe & Annison, 1969). This is biologically satisfactory for in such ruminants virtually all fat entering the small gut is in the form of free fatty acid (Leat & Harrison, 1969).

The α-glycerophosphate is mainly derived from tissue glucose but can-

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formed from free glycerol by the glycerokinase which is present in the intestinal mucosa (Haessler & Isselbacher, 1963; Saunders & Dawson, 1962). The other important enzyme present in the mucosa is a lipase specific for monoglyceride (Senior & Isselbacher, 1963). Thus it seems that under normal conditions when monoglyceride is available it is directly acylated. When there is an excess of free fatty acid in the cell the α-glycerophosphate pathway is also utilized. If, on the other hand, an excess of monoglyceride is present some of this is broken down by the specific lipase to provide fatty acids to esterify the remaining monoglyceride. There must be a requirement for these two systems and a specific lipase, unless the ratio of fatty acid to monoglyceride entering and passing through the cell is always 2:1 or unless large amounts of monoglyceride are to be found in the lymph or portal blood. Large amounts of monoglyceride are not found in lymph and portal blood and there is some evidence that monoglyceride and fatty acid are absorbed at different rates.

Once triglyceride has been resynthesized it is transported out of the cell either as a chylomicron or as very low density lipoprotein (V.L.D.L.) (Ochner *et al.*, 1969; Ochner & Jones, 1970). The relative importance of the two pathways depends on the fatty acid in the diet, saturated but not unsaturated fat being transported as V.L.D.L. The chylomicrons also contain phospholipid on their coat but although the pathways of phospholipid synthesis in the intestinal mucosa have been studied (Gurr *et al.*, 1965; Saunders *et al.*, 1968), little data is available about the importance of this moiety. On the other hand, the importance of the protein coat has been more extensively investigated. If protein synthesis is inhibited in the intestinal mucosa, either by puramycin (Sabesin & Isselbacher, 1965) or ethionine (Hyams, *et al.*, 1966; Kessler & Stein, 1969), triglycerides accumulate in the cell. This phenomenon seems to be best demonstrated in female animals, as has been the case in many examples of the experimental induction of fatty liver, and has its counterpart in man in the genetically determined disorder of abetalipoproteinaemia (Isselbacher *et al.*, 1964).

It would seem that although most of the absorbed short chain fatty acids pass unesterified through the intestinal cell and enter the circulation via the portal vein, long chain fatty acids are transported by the lymph after esterification. However it is probable that this is not an absolute rule and that a variable amount of long chain fatty acid can be transported by the portal vein. Thus, when fat is fed as an oil, in the absence of bile, a large amount of the absorbed fat does not appear in the lymph (Borgstrom, 1953; Gallagher *et al.*, 1965; Saunders & Dawson, 1963). This finding initially gave rise to the misconception that the absorption of triglyceride was totally dependent upon the presence of bile (Kim & Bollman, 1954).

Furthermore, when α-methyl fatty acids are absorbed they are probably transported partly via the portal vein (Blomstrand *et al.*, 1956), but when intestinal protein synthesis is impaired and lymphatic transport is blocked there is still a considerable overall absorption of fat which presumably occurs via the portal route (Kayden & Medick, 1969). Recently, using direct cannulation of the portal vein, Hyun and co-workers (1967) have shown that during normal fat absorption up to 15 or 20 per cent of the dietary long chain fatty acids may be transported by this route. The observations may partly explain the very large variation in the recovery of fed fat in lymph. The poor recoveries often reported need not necessarily be ascribed to poor technique or to the presence of portal lymphatic anastomoses which occur to a variable extent in different species and strains of animals (Dawson, 1967). The form in which the fat is transported in the portal blood is unknown but one would imagine it would most likely be fatty acid. If one feeds radioactive free fatty acid or triglyceride, a proportion is present in the lymph as the free acid, although the predominant labelled lipid will be esterified, indicating that a small fraction of the fatty acid has escaped esterification while passing through the absorptive cell. Such fatty acid would be equally available for transport by the portal vein or lymph and may well preferentially pass into the blood stream in view of the difference of the flow in the portal and lymphatic systems. The reason for this curious diversion of fat into the portal vein in the absence of bile is not clear. It can be completely overcome if the luminal fat is emulsified with a non-ionic detergent or bile salt before feeding. Thus, the suggestion that the non-appearance of fat in the lymph was due to sickness amongst the animals under study (Morgan, 1964) seems unlikely as animals in a comparable state of health could transport fat into the lymph when the lipid was emulsified (Gallagher *et al.*, 1965; Saunders & Dawson, 1963).

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THROMBOSIS IN THE DOG PRODUCED BY SINGLE RAPID INFUSIONS OF LONG CHAIN SATURATED FATTY ACIDS*

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Poole[33] showed, in 1955, that fatty acids shorten the clotting time of citrated plasma. In 1959, he[35] utilized Chandler's method[9] to study artificial thrombi. In 1961, with Connor[13], he showed that when straight chain saturated fatty acids with 16 or more carbon atoms were added to mixtures of blood in Chandler's apparatus, solidification of blood was accelerated and these solid masses were long. The solidification had the gross and microscopic appearance of a thrombus. Such changes did not occur when short chain fatty acids or unsaturated fatty acids were used. They stated that such a mechanism may operate in the body and, therefore, deserves serious consideration. Subsequently, Connor[10] extended these *in vitro* observations with the use of human blood and with additional fatty acids. Their work stimulated us to determine whether intravenous infusions of long chain saturated fatty acids could produce thrombosis in the living animal (dog) and, if so, what effects these acids had on *in vitro* tests of coagulation and the blood lipids.‡

Method and Material. SUBJECTS. All experiments were done on apparently healthy male mongrel dogs weighing an average of 10 kg. The dogs were fed a standard diet of Gaines Dog Chow and on the morning of testing were fasting for 12 hours. For the infusions the dogs were anesthetized with Pentobarbital.

I. TECHNIQUE. *Intravenous.* The femoral vein was entered with a #17 gauge Monocote (Armour Laboratories)¶ needle through which a plastic catheter§ tube was directed into the inferior vena cava. Samples of blood were obtained either by allowing the blood to run freely through the catheter into either a siliconized ‖ or polyethylene tube or by gentle aspiration with siliconized (2-syringe technique) or disposable plastic syringes.# Maximum effort was made to avoid glass contact or any activation of Factor XII (Hageman) or VI (PTA). Sufficient blood was obtained for baseline lipid and coagulation studies; these are called *pre-infusion* values. The fatty acid solution was then injected through the tubing over a 5-minute period.

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‡A preliminary report was published in Clin. Res., 11, 192, 1963. Identical results were reported by Connor, Hoak and Warner[11], in the same year.
We postponed our full report until the termination of our chronic experiments.
¶Monoject Disposable needles, Roche Products Company, Waterbury, Conn.
§Desert Intracath, C. R. Bard, Inc., Murray Hill, N. J.
‖Drifilm, General Electric.
#Pharmaseal-Stylex Expensable Syringes, Pharmaseal Labs., Glendale, Calif.

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(Experiments were done to show that the time taken, if between 3 to 20 minutes, for the infusion had no effect upon the end results.) Three minutes after the infusion, samples were obtained and the same studies performed as had been done prior to the infusion. These are called *post-infusion* values. In one group of dogs, blood was obtained from the jugular vein for analysis when prolonged observations were made.

In the event of death of the animal, an immediate necropsy was performed in the first 37 dogs infused. A jugular vein was ligated and examined for clots within 45 minutes after death in 10 dogs, according to Wessler's technique[40].

II. REAGENTS. The fatty acids used were all commercially available and of the purest quality*.

The acids were converted to their sodium salts at a concentration of 0.1% suspension by gentle warming with sodium hydroxide. The pH was then adjusted to 8 with N/10 HCl. Saline with adjusted pH and molarity was used as a control. Connor and Poole[19] showed no difference in *in vitro* thrombogenesis between this saline and distilled water, 5% bovine albumin, sodium chloride of molarity and pH comparable to the NaCl contained in the fatty acid solution. The solutions were all opalescent, oleic acid being the most, linolenic the least.

The fatty acids were given in doses of 10 ml. per kg. of a 1 in 1000 W/V solution. These were pre-heated to 60° C., the temperature of greatest solubility, then cooled to 40° C. and infused at 37° C. When mixtures of acids were administered, they were prepared to contain twice the concentration of fatty acids with the same normality as that of each separate acid and the final solution was the same (a 0.1% solution). The kaolin was obtained from commercial sources† and was given as a 1:100 W/V solution in physiological saline.

III. ANALYSIS OF SAMPLES. *Coagulation Techniques.* All dogs were fasted overnight prior to the obtaining of samples. Blood was collected from each animal either by allowing it to run directly into a plastic or siliconized test tube from a plastic catheter or through the use of a two-syringe technique, as mentioned above. Thirty milliliters of blood were obtained before and after infusions. The blood obtained was distributed as follows: 1.0 ml. was placed in each of 3 glass tubes and 3 silicone tubes of 10 mm. internal diameter for clotting time determinations; 4.5 ml. were added to 0.5 ml. of 0.1 molar sodium oxalate to obtain platelet-rich plasma for prothrombin determinations; 9 parts of blood were added to one part of 3.8% sodium citrate; 2 ml. were placed in each of 2 glass tubes for performance of the prothrombin consumption; and 5.0 ml. were added to a tube containing 7 mg. of EDTA‡. For platelet-rich plasma blood was centrifuged at 1500 rpm for 15 min. in a PR2 International Refrigerated Centrifuge at 4° C.

Coagulation Methods. The majority of the methods used in these laboratories have been previously reported (Day and Zarafonetis[15]) and are standard with some modifications.

Whole Blood Clotting Time in Glass. Three glass tubes in water bath at 37° C., each containing 1 ml. of blood. The tubes first are tilted after 2 minutes and thereafter at 30 second intervals.

Whole Blood Clotting Time in Silicone. Method of Merskey and Vossel[28] with 3 tubes being used instead of 6.

Recalcification Time. Method of McDonald and Fullerton[29]: 0.2 ml. of plasma was recalcified with 0.2 ml. volume of M/50 calcium chloride. The recalcification time was done in triplicate and the mean recorded.

One-stage Prothrombin Time. Method of Quick[30]. A commercial source of thromboplastin was used.¶

Prothrombin Consumption Time. Serum for this test was obtained by allowing 2 ml. of whole blood to clot at 37° for 1 hour. The clot was then removed from the tube and the serum incubated for another 30 minutes. This was then tested for residual prothrombin activity.

Clot Retraction. Whole blood (1 ml.) in siliconized tubes at 37° C. for periods of 1 and 4 hours. Test was done in duplicate.

Stypven Time.§ Russell Viper Venom Test was done both on whole plasma and diluted plasma according to the method of O'Brien[31], except that the immediate technique of Maclagen, Billimoria and Curtis[26] was used. The plasma concentrations are referred to as 1:1 for *whole plasma* and 1:4 for *plasma diluted with saline.*

Hicks-Pitney Modification of the Thromboplastin Generation. This test was done as described by Hicks and Pitney[32]. A commercial cephalin was used as a source of platelet substitute.#

Partial Thromboplastin Time. Done by the method of Rodman, Barrow and Graham[41]. A commercial cephalin was used as a source of platelet substitute.#

*Nutritional Biochemical, Cleveland, Ohio.
†China Clay, Braun Chemical Co.
‡Vacutainer, Becton, Dickinson and Company, Rutherford, N.J.
¶Simplastin, Warner-Chilcott.
§Stypven, Burroughs Wellcome & Co.
#Platellin, Warner-Chilcott.

Platelet Count. Both the direct phase contrast method of Becker and Cronkite[2] and a modification of the new methylene blue stain[10] (Becker[1]) for an indirect method were used. Venous blood served as the source of blood supply for this and all other hematological techniques.

CHEMICAL METHODS. The serum cholesterol and cholesterol esters were determined by the method of Zak[15,16] as modified by Leffler[7]. The phospholipids were determined by the method of Youngburg and Youngburg[17] as modified by Sunderman and Sunderman[4]. Free fatty acids in the serum were measured by the methods of Dole[13] and Gordon[12]. The serum lipids were extracted by the method of Sperry and Brand[14] as modified by Bragdon[7]. Total lipids in serum and triglycerides by difference were evaluated by the method of Bragdon[8], from a modification of Bloor's method[3].

Results. Table 1 analyzes the mortality after single intravenous infusion of each fatty acid. It can be seen that the two unsaturated fatty acids (oleic, C 18:1 and linolenic, C 18:3) failed to kill any dog, whereas the two long chained saturated fatty acids (stearic, C 18:0 and palmitic, C 16:0) killed respectively 25 and 33% of the dogs. This difference in mortality is significant. Three of 5 dogs from each group of animals receiving saturated fatty acids were found by the Wessler technique to have thrombi in the jugular veins, and one from each group of 3 dogs receiving unsaturated fatty acids had similar findings.

Table 2 shows that 8 of 10 stearic-infused and 8 of 11 palmitic-infused dogs had, at necropsy, massive venous and arterial thrombi. No thrombi were found in the dogs given saline. However, the Wessler technique revealed thrombi in one dog given oleic acid and one dog given linolenic acid. Kaolin produced thrombi indistinguishable from those found in the dogs infused with saturated fatty acids.

Table 1 shows that a mixture of oleic and palmitic acids produced only a 10% mortality. This reduction suggests that oleic acid produces partial protection against the lethal effect of palmitic acid; on the other hand, oleic acid does

TABLE 1.—MORTALITY ASSOCIATED WITH INTRAVENOUS INFUSIONS OF FATTY ACIDS IN DOGS

Acid	*No. of Dogs Infused*	*Lived*	*Died*	*Mortality %*
Oleic	29	29	0	0
Linolenic	20	20	0	0
Stearic	36	27	9	25
Palmitic	45	30	15	33
Oleic-Palmitic	20	18	2	10
Oleic-Stearic	28	8	20	71
Saline	17	17	0	0
Totals	195	149	46	

TABLE 2.—INCIDENCE OF GROSS THROMBOSIS FOUND AT NECROPSY AS THE RESULT OF INTRAVENOUS INFUSIONS OF FATTY ACIDS

Acid	*Necropsied*	*Thrombus Found*	*No Thrombus Found*
Oleic	3	0	3
Linolenic	3	0	3
Stearic	10	8	2
Palmitic	11	8	3
Oleic-Palmitic	2	2	0
Oleic-Stearic	4	4	0
Saline	2	0	0
Kaolin	2	2	0

TABLE 3. MEAN CHANGES NOTED IN COAGULATION TESTS FOLLOWING INFUSION OF FATTY ACID SOLUTION INTO DOGS

Acid	Coag. Time Glass Min.	Coag. Time Silicone Min.	Recal. Time Sec.	Serum Pro. Time Sec.	Plasma Pro. Time Sec.	Part. Thromb. Time Sec.	Hicks-Pitney TGT Sec.	Stypven Time (1:1) Sec.	Stypven Time (1:5) Sec.	Platelet Count
Oleic C-18:1	0.187 *16	0.91 11	1.25 16	1.3 16	−0.31 15	+0.02 10	+0.06 15	+0.22 15	−0.020 15	223,400 8
Linolenic C-18:3	0.9 20	1.8 18	0.8 19	−10.5 19	0.05 20	+0.58 18	+1.5 4	+0.5 19	−1.3 20	135,000 20
Palmitic C-16:0	0.26 23	3.6 18	−18.0 17	−20.2 21	−1.6 19	−3.5 12	+0.26 19	−3.6 13	−3.8 16	−197,000 11
Stearic C-18:0	−0.08 12	−1.8 11	−15.0 14	−25.2 14	+0.32 15	+5.0 8	+0.83 12	−3.5 14	−3.8 14	−56,750 8
Oleic-Palmitic	−1.6 21	−3.2 24	−19.2 25	−20.3 23	−1.6 19	+1.66 9	−0.36 11	−3.7 26	−0.4 23	−177,707 14
Oleic-Stearic	−8.0 7	−12.8 5	−30.8 6	−30.5 9	+0.23 9	+2.25 6	+0.66 3	−1.4 9	−0.85 9	−197,000 6
Saline	−0.17 18	−1.2 17	+2.5 16	+0.3 16	+0.08 17	−0.40 16	−0.4 5	+0.43 17	1.73	84,125 16

*Figure in this position indicates number of paired observations made for the fatty acid concerned.

not protect against stearic acid (mortality 66%). Massive thrombosis was present in all these dogs that died.

COAGULATION EFFECTS. Table 3. These were done as paired experiments only on those animals who survived the procedures, because those animals that died did so within a few minutes after the infusions, making it impossible to obtain *post-infusion* blood samples due to blockage of the tip of the catheter.

In the series of paired experiments, all the groups of animals were studied completely a total of 123 times. Due to technical problems, such as difficulty in drawing blood, not all the results were acceptable and had to be eliminated. Generally, each acid had between 11 to 25 pairs of determinations. These were divided among the various acids as given in Table 3. This shows that the number of observations made on oleic acid was 16, linolenic 20, palmitic 23, stearic 14, oleic-palmitic 25, oleic-stearic 9, and saline controls, 16. Fewer paired observations were obtained on the oleic-stearic mixtures due to its lethality and these results are not strictly comparable due to dissimilar numbers. The mean of the sum of the difference is given in Table 3. (In interpretation of the results, it must be remembered that we were infusing 10 ml. per kg. into a dog whose blood volume is 92 ml. per kg. and a slight dilutional effect occurred due to an increase in blood volume, note the saline control. This would have caused a dilution of any of the coagulation proteins seemingly producing a prolongation of the times obtained in the coagulation tests. As most of the results showed no change or an actual shortening of the times, these changes are even more significant. The results must be interpreted in this light.)

Coagulation in Glass. It may be seen from these experiments that very little effect was produced on the Lee-White coagulation time in glass by saturated, unsaturated or combination of unsaturated and saturated fatty acids or saline. The fact that all showed a negative value is of interest, but the amount of shortening is certainly not sufficient to be significant in any one particular instance when compared with the control.

Coagulation in Silicone. All acids and saline produced a shortening of the silicone clotting time; however, the greatest changes were produced by the combination of oleic-stearic acids, oleic-palmitic, and palmitic acids. There was no difference between the shortening seen with linolenic and stearic acids. The amount of shortening is not significant when one compares the effect of stearic with oleic acid but is significant between oleic and palmitic.

Recalcification Time. All acids produced a shortening of the recalcification time in glass, while saline increased it. The greatest shortening was seen with the saturated acids, palmitic and stearic acid, whether alone or in combination. Oleic-palmitic acid caused the same amount of shortening as did palmitic acid alone, while oleic-stearic acid produced the greatest overall change. These results seem to show a definite decrease in the recalcification time after the infusions of saturated fatty acids.

Serum Prothrombin Time. There was a decrease in the serum prothrombin time at the end of 90 minutes in all groups of dogs except those receiving saline alone. The serum prothrombin time in all dogs prior to infusions was always above 25 seconds, and at the end of 90 minutes was usually shortened in those receiving saturated acids. There was some shortening in the oleic and linolenic acid groups, but the greatest amount seen was in the groups receiving the saturated fatty acids. This is almost twenty times that seen in the control and oleic group and two times the amount of shortening seen with the linolenic group. This appears to be independent of any changes on the platelets (see below). This reflects a lack of consumption of prothrombin brought about by the infusion and may reflect

either an anticoagulant-like effect or possibly a thrombin-like action.

Stypven Times. There did not seem to be any difference in the changes produced in the Stypven-plasma clotting times between the undiluted plasma (1:1) test, and the diluted (1:4) plasma tests. Oleic and linolenic acids and the saline produced negligible changes in these values with actual plus values in most cases. Both palmitic and stearic acid produced a definite shortening of the various Stypven times. The combinations of oleic acid and palmitic acid and stearic acid produced the same amount of changes although the latter were not as great as the others. These values seem to indicate no severe factor V drop, as factor V is necessary for the performance of this test.

Platelet Counts. All acids and saline produced a definite drop in the platelet count. However, these did not always fall to so-called bleeding levels (below 50,000). There was a definite difference in these studies which can be seen. Saline, stearic and linolenic acid produced the least severe thrombocytopenias; whereas oleic, palmitic and their combinations produced the greatest thrombocytopenia.

Clot Retraction. No lack of clot retraction was noted in any specimen. The clots were not as well retracted in some as in others, but we are unable to decide at this time if there are any significant differences.

One-stage Prothrombin Time. Table 3. There was no change produced in the one-stage prothrombin time by the infusions of the various acids.

Hicks-Pitney Modification of Thromboplastin Generation. Table 3. No changes were noted in this test on any acid tested. This would seem to argue against factor VIII decrease.

Partial Thromboplastin Time. Table 3. No significant difference could be noted between the effects of the various acids upon the partial thromboplastin time. As this test is sensitive to all factors in coagulation except platelets and factor VII, it would seem to confirm the notion that no changes occurred as a result of a decrease in coagulation factors.

The Blood Lipids. Table 4. These were studied in a total of 101 paired experiments. When compared with saline, no significant change after the infusion was noted in the total cholesterol, free cholesterol, cholesterol esters, total lipids, phospholipids or triglycerides. The least change occurred in the oleic, palmitic and their combination's effect upon the nonesterified fatty acids, but these could not be correlated with any other lipid or coagulation value. Nor did they correlate with the mortality statistics. These values seem to indicate that the thrombosis produced was independent of any changes in the blood lipid values, especially any great increase in the nonesterified fatty acid proportion of the blood despite the administration of an amount designed to raise the plasma concentration by 10%.

Discussion. These studies indicate that saturated fatty acids, in contrast to unsaturated ones, are thrombogenic and frequently fatal when infused intravenously into dogs over a 5-minute period in doses of 10 ml. per kg. as a 0.1% suspension of their salts. Connor *et al.*[11,12,23] have reported similar findings in a similar group of dogs and our results would seem to confirm their findings. The amount of fatty acid injected into our animals was not sufficient to cause a measurable rise in the nonesterified fraction in the blood.

The mechanism whereby saturated fatty acids promote thrombosis is not clear. Satisfactory specimens of blood for coagulation studies could not be obtained because of the fact that the catheter or needle was often impaled upon solid or gelatinous blood and suitable specimens were not available for analysis. It was for this reason that we analyzed the characteristics of blood from dogs who survived infu-

TABLE 4.—MEAN CHANGES PRODUCED IN LIPID LEVELS BY THE INTRAVENOUS INFUSION OF FATTY ACID SOLUTIONS INTO DOGS

	Total Chol. mg./100 ml.	*Free Chol. mg./100 ml.*	*Ester. Chol. mg./100 ml.*	*Esters Chol. mg./100 ml.*	*Phospho-Lipids mg./100 ml.*	*Tri-Glycer. mg./100 ml.*	*Total Lipids mg./100 ml.*	*Non-ester. F.A. μEq./L.*
Oleic C-18:1	—8.0 14	—6.3 14	—0.5 14	—3.0 14	—16.2 14	—7.0 14	—26.5 14	17.0 16
Linoleic C-18:2	—14.6 9	—7.1 10	—7.4 9	—12.6 9	—28.6 9	—6.3 9	—55.2 9	219.4 10
Palmitic C-16:0	—10.0 17	—2.9 17	—7.5 17	—6.0 16	—27.7 18	+0.125 18	—47.4 18	19.4 20
Stearic C-18:0	—9.0 13	—8.2 13	—4.4 13	—5.1 13	—30.0 13	—1.7 13	36.4 13	146.3 9
Oleic-Palmitic	—7.4 16	—1.8 16	—4.6 16	—10.4 16	—27.4 15	—6.1 15	34.4 16	5.9 16
Oleic-Stearic	—6.0 11	—4.0 11	—3.4 11	—4.7 11	13.6 11	17.0 11	31.0 11	66.5 11
Saline	—15.4 15	—3.4 15	—12.0 15	—19.0 15	—36.9 15	—6.4 15	—50.5 15	187.4 14

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sions with saturated fatty acids and compared the findings to blood from dogs given unsaturated fatty acids and saline. It was hoped that such indirect evidence might provide clues to the thrombogenic characteristics of blood after contact with saturated fatty acids, and thereby correlate *in vivo* thrombogenesis with *in vitro* coagulation tests.

The coagulation studies seem to provide *in vitro* evidence that the effect produced in those animals which received saturated fatty acids is different from those which received unsaturated acids. The most significant change that occurred was in the recalcification time. That this test was able to show such a dynamic change was of interest to us in that it would seem to support the value of this procedure in defining the "hypercoagulable" state as has been mentioned by Fullerton and McDonald[20]. Poole[33] showed the clot-acceleration of recalcified plasma due to saturated and unsaturated fatty acids *in vitro*.

The clotting time done in glass tubes showed no difference between the various acids, but the clotting time done in silicone and in the presence of Stypven tended to show a shortening with infusions of saturated fatty acids but these changes are not as dramatic and are of questionable significance. The "Stypven time" has been regarded by some as a "test of lipemia and not of coagulation" (Poole[34]); yet we noted no changes in any of the lipid fractions of the blood which we measured. Others have challenged the idea that hyperlipemia alone is responsible for the change in coagulation time of plasma, tested with Russell Viper Venom (Zak *et al.*[39]). Esnouf and Williams[18] demonstrated that the venom reacts with factor X to form an esterase which activates prothrombin in the presence of factor V and phospholipid. Mustard *et al.*[29] demonstrated a shortening of the Russell Viper time after an intravenous injection of phosphatidyl serine into dogs and yet this was associated with an anticoagulant effect on other clotting tests as well as on thromboplastin generation. This seems to point out that shortening of the Stypven time is probably directly related to an action of the phospholipid (or fatty acid) and is independent of the other lipids. Maclagan, Billimoria and Curtis[8] showed no relationship between shortened Stypven times and unesterified fatty acid levels in humans given animal or vegetable fat meals.

The exact reason for the shortening of the serum prothrombin time is not clear at this time as it would seem to indicate a lack of reactants which are necessary for the first stage of coagulation which is hard to correlate with the minor changes in the Hicks-Pitney and Partial Thromboplastin times. With minor exceptions, these tests measure the same reactants except that the latter two tests are insensitive to changes in the platelets, either quantitative or qualitative, as they make use of a platelet substitute and thereby avoid platelet changes. We did note a fall in the total platelet count with all substances (partially dilutional—note saline) which could not be correlated with the changes in the serum prothrombin time as might be seen in the values produced by oleic acid which caused the least shortening of the serum prothrombin time, yet was able to cause some of the greatest falls in platelets. Several other explanations might be advanced. One might suggest that the fatty acids might "coat" the platelets so that they are incapable of functioning or releasing their thromboplastic material. Another thought might be that the consumption of prothrombin might be inhibited by the production of a thrombin-like material. In our experiments[14] on hyperlipemic human patients, we have noted absence of adequate prothrombin consumption on several occasions with known reason. This phenomenon is noted in other such

published series (Mersky and Vossel[28], Peyman *et al.*[32]), although it is not a regular phenomenon.

The changes produced in the coagulation tests might be those which could be expected if one assumes that the fatty acids tested, especially the saturated fatty acids, have the propensity to activate the Hageman-PTA system and produce activation product. The fact that the dogs which received Kaolin (a potent activator substance of the Hageman-PTA system) had the same gross thrombi as those receiving saturated fatty acids would lend support to this thesis. Siese, Muschos and Becker[42] point out that all clotting factors are already present in excess in the normal person and it can hardly be expected that a shortening of the clotting time can indicate anything except the presence of a circulating thromboplastic substance or activation of the Hageman-PTA system. Connor[10] and Margolis[27] have felt that the negative charge of the micelles of fatty acids function to produce activation of Hageman factor (factor XII). Similar thoughts have been advanced by Didisheim[17] and others (Connor[10], Ratnoff[39]). However, recent experiments from this laboratory (Soloff and Wiedeman[43]) have shown that, in the bat, the thrombogenic effect of saturated fatty acids cannot be duplicated by large doses of kaolin. This casts some doubt on the concept implicating activation of Hageman factor. Furthermore, Botti and Ratnoff[4] have shown that the long chain saturated fatty acids act not upon a purified preparation of Hageman factor, but upon both inactive and active plasma thromboplastin antecedent (PTA factor XI), thereby circumventing the supposed action of Hageman factor (XII). Ratnoff has shown that the ellagic acid is capable of causing factor XII activation (Botti and Ratnoff[5], Ratnoff and Crum[40]).

The effect on the coagulation of the blood by other factors known to raise the free fatty acid concentration of blood has been reviewed by Poole[36] and Poole and Frank[37] who note the association between electrical stress in the rat causing an elevation of the fatty acids and shortening of the whole blood clotting times as have been previously published (Delong, Uhley and Friedman[16]). The same association is noted between the elevation of free fatty acids produced by the injection of Adrenalin (Dole[18]) and shortened whole blood clotting times (Cannon and Gray[8]). Mustard *et al.*[30] call attention to the same association between the Adrenalin and shortening of the clotting times seen in their extracorporeal shunt experiments and speculate whether this may be due to the effect on the mobilization of free fatty acids or to an *in vitro* effect on the blood clotting mechanisms. Hoak, Poole and Robinson[21] have recently produced small thrombi with the Wessler technique through the injection of ACTH and Adrenalin, among other substances. These findings are of special significance because they appear to indicate that fatty acids apparently usually bound to albumin may also be thrombogenic under circumstances in which their concentration rises abruptly and sufficiently high.

The fatty acid concentration of the blood was not measurably increased after their infusions. This finding suggests that excess fatty acids have disappeared from the circulating blood by the time thrombosis has occurred. Therefore, a normal free fatty acid concentration of blood, when thrombosis is clinically evident, does not exclude the possibility that increased amounts of free fatty acids may have produced such a pathological state.

Summary. This study was undertaken to determine whether long chain saturated fatty acids, which are known to enhance solidification *in vitro*, can produce thrombosis *in vivo*.

One hundred and ninety-five dogs were used. Each was infused intravenously over a 5-minute period with 10 ml. per kg. of a 0.1% suspension of the sodium salt of oleic, linolenic, stearic or palmitic acid and also a combination of oleic-palmitic and oleic stearic. Saline was used as a control.

Oleic acid, linolenic acid and normal saline were innocuous. Twenty-five per cent of stearic-acid infused dogs, 33% of palmitic, 10% of oleic-palmitic and 71% of oleic-stearic infused dogs died. Massive thrombosis (venous and arterial) was found at necropsy in over 80% of these dogs. In those that survived the infusion, immediate necropsy after the Wessler technique frequently showed small localized thromboses.

The only laboratory test significantly different from the normal was the recalcification time which was shortened in dogs given the saturated fatty acids.

The significance and possible mechanism of these results are discussed.

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SUMMARIO IN INTERLINGUA

Thrombose Producite in Canes per un Sol Rapide Infusion de Saturate Acido Grasse a Longe Catenas

Le presente studio esseva interprendite con le objectivo de determinar si o non saturate acidos grasse a longe catenas (le quales cognoscitemente promove solidification in vitro) pote producer thrombose in vivo.

Esseva usate 195 canes. Cata-un recipeva un infusion intravenose, de un duration de 5 minutas, de 10 ml per kg de peso corporee de un suspension de 0.1 pro cento del sal a natrium de acido oleic, linolenic, stearic, o palmitic o etiam de un combination del sales a natrium de acido oleic e palmitic o oleic e stearic. Solution salin esseva usate como substantia de controlo.

Acido oleic, acido linolenic, e solution salin normal esseva innocue. Vinti-cinque pro cento del canes recipiente infusiones de acido stearic, 33 pro cento de illo recipiente acido palmitic, 10 pro cento recipiente acido oleic e palmitic, e 71 pro cento recipiente acido oleic e stearic moriva post le infusion. Massive thrombose (venose e arterial) esseva trovate al necropsia in plus que 80 pro cento de iste canes. In le superviventes, necropsia immediate con le uso del technica de Wessler monstrava frequentemente minor thrombose localisate.

Le sol test laboratorial con resultatos singificativemente anormal esseva le test del tempore de recalcification. Iste tempore esseva reducite.

Le signification e le mechanismo possibilemente responsabile de iste phenomenos es commentate.

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ACTIVATION OF TESTOSTERONE BY HIGHER FATTY ACIDS AND THEIR ACID SODIUM SALTS*

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(Received for publication, October 1, 1937)

The influence of male sex hormones on the development of the accessory sex organs in the rat can be enhanced: (*a*) by esterification with certain lower fatty acids (1, 2); (*b*) by administering the hormone together with either certain higher fatty acids (3) or high molecular weight saturated primary alcohols ((3) p. 1974); the injection of the hormone and the second component must be made at the same time and site to obtain the desired effect. It has further been found that the free hormone only can be activated, and that a combination of hormone esters with fatty acids appears to bring about no further increase of the physiological effect ((1) p. 1986, (2)).

Laqueur and his collaborators found that an activating factor, the so called *X* substance, is present in various tissues (4). Tschopp and coworkers succeeded in isolating palmitic acid from the *X* substance fraction of testes by means of the chromatographic adsorption technique (5). They also investigated the activating effect of a number of naturally occurring fatty acids and found palmitic acid to be the most effective (3).

In an earlier paper Ehrenstein and Britton (6) describe the isolation of an acid sodium salt of palmitic acid, $C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$, from adrenal glands. Its possible action as an activator of male sex hormones is there discussed. Evidence has now been obtained that this salt is not originally present in the adrenal tissues. It is apparently formed by the permutit treatment employed during the preparation of corticoadrenal

* Grateful acknowledgment is made of aid received from the Rockefeller Foundation.

extract. Moreover, it was observed that permutit can generally be used for the isolation of higher fatty acids from various kinds of tissue. Thus we obtained the acid sodium salt and, by acidification, palmitic acid from testes and liver. Only traces could be isolated from ovarian tissue. This indicates that the occurrence of free palmitic acid, like that of the *X* substance, is not restricted to the tissues of the endocrine glands.

The present paper deals especially with the activating effect of palmitic and stearic acids and their corresponding acid sodium salts on the male sex hormone testosterone.

EXPERIMENTAL

Chemical Procedures

Isolation of Palmitic Acid from Various Tissues—All tissues were treated according to the modified method used in this laboratory for the preparation of corticoadrenal extract. Before the treatment with permutit the alcoholic solution was brought to dryness. On addition of pure acetone to the residue, a sticky precipitate was obtained, apparently consisting mainly of those last traces of phospholipids which had escaped the first precipitation. On acidification of this resinous material, which contained but traces of sodium, no free fatty acid could be isolated. The acetone solution was brought to dryness, the residue dissolved in 70 per cent alcohol, and this alcoholic solution treated with permutit in the usual manner. This solution was then brought to dryness and the residue treated with acetone. In all experiments (beef adrenals, testes, ovaries, liver) a more or less powdery, light brown precipitate was obtained which, on being treated with water, yielded a white precipitate of microscopically fine flat needles. These needles represented the acid sodium salt of palmitic acid, $C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$, in a more or less pure form. On acidification, the free acid was obtained and could be readily identified as palmitic acid. A satisfactory yield was obtained from adrenal glands; testes and liver yielded a little less and not quite pure palmitic acid. Only traces of rather impure palmitic acid could be secured from ovarian tissue.

Preparation of Sodium Bipalmitate, $C_{15}H_{31}COOH \cdot C_{15}H_{31}COONa$, and Sodium Bistearate, $C_{17}H_{35}COOH \cdot C_{17}H_{35}COONa$—0.02 mole of pure acid (Kahlbaum) was dissolved in 80 cc. of warm 95 per cent alcohol. To this solution was added rather quickly a warm solution of 0.01 mole of sodium hydroxide in 57 cc. of alcohol (the NaOH is first dissolved in 2 cc. of water, then 55 cc. of 95 per cent alcohol are added). On standing, the salt crystallized in long flat spears. The melting point of the bipalmitate was 122–124° (sintering between 80–90°) and that of the bistearate 121–124° (sintering at about 100°). It should be noted that the molten salts were not quite transparent.

Animal Experiments

Method of Assay—Young, male white rats weighing 50 to 80 gm. were castrated. They were used for assaying the various preparations not before the 25th day and not later than the 30th day after operation. Subcutaneous injections were carried out for 9 successive days, and sacrifice of the animals and dissections were made on the 10th day. The daily dose was always dissolved in 0.5 cc. of sesame oil. In those cases in which testosterone was combined with either a free acid or an acid sodium salt, the two constituents were dissolved in 0.25 cc. of sesame oil each. The syringe was filled by drawing in the solution of testosterone first and that of the activator thereafter. Since palmitic acid and stearic acid as well as their corresponding acid sodium salts are only slightly soluble in sesame oil at room temperature, these preparations were heated in a paraffin bath to about 130° (*i.e.* a little higher than the melting points of the salts). Although the free acids yield a homogeneous solution at a considerably lower temperature, they were heated as high as the salts in order to insure comparable conditions. It should be stated that with the higher concentrations of acid, and especially of salt, a formation of lumps under the skin was observed. The health of the rats was not, however, impaired; all experimental animals appeared to be in good condition at the time of sacrifice.

Results

By comparison of the results in Tables I and II, it will be seen that under the experimental conditions described, the effect of testosterone on the development of the seminal vesicles and prostate gland can be considerably enhanced by combining the hormone either with free higher fatty acids (palmitic acid, stearic

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acid) or their corresponding acid sodium salts ($C_{15}H_{31}COOH$.. $C_{15}H_{31}COONa$, $C_{17}H_{35}COOH \cdot C_{17}H_{35}COONa$). Rather low doses (up to 25 mg. daily) of the salts proved to be much more effec-

TABLE I

Activation of Testosterone

50 micrograms of testosterone and various concentrations of activator were administered daily to castrated rats.

No. of rats	Concentration and kind of activator	Average body weight at killing	Average weight: Seminal vesicles	Average weight: Prostate	Seminal vesicles	Prostate
	Palmitic acid					
	mg.	gm.	mg.	mg	per cent of body weight	per cent of body weight
4	25 Palmitic acid	145	51	46	0.034	0.031
4	50 " "	156	154	86	0.100	0.055
4	10 Na bipalmitate	146	92	66	0.060	0.045
6	25 " "	135	139	75	0.104	0.055
6	50 " "	126	185	96	0.147	0.075
	Standard deviation*..	12.1	36.5	14.6	0.0293	0.0112
	Stearic acid					
4	25 Stearic acid	158	70	45	0.044	0.028
4	50 " "	166	175	100	0.106	0.061
4	75 " "	177	311	121	0.176	0.068
4	10 Na bistearate	176	110	49	0.062	0.028
4	25 " "	156	157	89	0.102	0.057
4	50 " "	153	144	87	0.095	0.057
4	75 " "	152	135	91	0.099	0.060
	Standard deviation*..	9.2	41.0	11.4	0.0263	0.0072

The figures contained in the table confirmed preliminary experiments carried out with fewer animals.

* Pooled standard deviation of observations within groups from their respective means.

tive than the same doses of the free acids. With medium doses (50 mg. daily) the effect was about the same with either the free acid or the acid sodium salt. Higher doses of free acid (75 mg. daily), as shown in the stearic acid series, brought about a further

increase of the activating effect. Activation with the acid sodium salt, however, could not be increased beyond that obtained with the 50 mg. daily dosage.

TABLE II

Effectiveness of Testosterone, Testosterone Acetate, and Testosterone Propionate†*

Castrated rats were given 50 micrograms of the various preparations daily, no activator being added.

In the case of control animals which were given only 0.5 cc. of pure sesame oil daily, the average weight of the seminal vesicles was 5 mg. and of the prostate 3.9 mg. (2 animals).

No. of rats	Concentration of male hormone in 0.5 cc. sesame oil	Average body weight at killing	Average weight: Seminal vesicles	Average weight: Prostate	Seminal vesicles	Prostate
		gm.	mg.	mg.	per cent of body weight	per cent of body weight
5	Normal animals; no injection given	150	205	110	0.134	0.072
	Standard deviation‡		67.1	34.7	0.0347	0.0182
4	50 micrograms testosterone	140	25	21	0.018	0.016
3	50 " " acetate	164	83	54	0.056	0.037
4	50 micrograms testosterone propionate	158	62	34	0.041	0.021
	Standard deviation‡	20.4	21.2	9.0	0.0197	0.0066

* Perandren, a preparation of the Ciba Company, Inc., New York. By courtesy of Dr. Haskell.

† Testoviron, a preparation of the Schering-Kahlbaum, A. G., Berlin. By courtesy of Professor W. Schoeller.

‡ Pooled standard deviation of observations within groups from their respective means.

A satisfactory explanation of the action of the salts cannot be given at this time. Possibly the suspension of the salt in sesame oil, which represents a mass of vaseline-like consistency, acted as a colloidal system such that if 25 mg. or more of the salt was administered daily all of the testosterone was adsorbed by it and slowly liberated at a more or less constant rate.

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The activity of highly effective esters of testosterone (such as the acetate and propionate) was considerably surpassed in these experiments. Furthermore, the weights of the accessory sex organs of normal animals of approximately the same body weight were exceeded in the experiments in which 50 micrograms of testosterone were combined with 75 mg. of free acid.

It is known that testosterone palmitate and stearate ((1) p. 1980) are almost inactive. Thus the suggestion of an ester theory for the explanation of the activating effect of free higher fatty acids is not justified.

SUMMARY

The increased effect of testosterone upon the accessory sex organs of the male rat induced by palmitic or stearic acid or by their acid sodium salts is demonstrated by comparison with the effect of testosterone alone or with that of two esters of testosterone.

The authors feel much indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for the generous supply of testosterone.

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THE INTERACTION OF HUMAN ERYTHROCYTES WITH SODIUM PALMITATE

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Evidence has recently been acquired that the unesterified fatty acids (UFA) present in human blood plasma are of great metabolic significance, apparently functioning as a transport form of lipid readily available as a substrate for oxidation. This evidence includes studies of UFA arteriovenous differences and studies of the responsiveness of plasma UFA concentration to changes in dietary status and to the administration of hormones (1–3). Furthermore, turnover studies have demonstrated an extremely rapid turnover of this lipid fraction. After intravenous injection of C^{14}-labeled UFA, plasma activity declined with initial half-times of two to three minutes in the fasting human subject (4, 5), and with even shorter half-times in the rat (6) and the dog (7). In these studies, labeled CO_2 was found in expired air within minutes after the injection of labeled UFA and, in the human subject, reached its peak specific activity in about 30 minutes (5).

Quantitative investigations of the interaction of unesterified fatty acids with human serum albumin and with human lipoproteins have revealed the fatty acids to be tightly bound by these proteins, particularly by albumin (8, 9). On the basis of albumin binding alone it has been estimated that in normal human plasma less than 0.01 per cent of plasma UFA exists free (unbound) in solution (8). The problem therefore emerges of the mechanism whereby plasma UFA, which exists tightly bound to serum albumin, is rapidly transported to intracellular loci of oxidation (mitochondria), with the extremely rapid turnover described above. One suitable hypothesis would be to postulate the existence of receptor sites on cell surfaces which are able to bind UFA tightly enough to compete with serum albumin. Such sites, if capable of exchanging UFA rapidly, could serve as intermediates in the transfer of plasma UFA from serum albumin to intracellular loci. In order to test this hypothesis the interaction of human erythrocytes with a long-chain fatty acid salt, sodium palmitate, has been studied by measuring the distribution of the fatty acid between erythrocytes and serum albumin. The results of these studies are reported herein.

MATERIALS AND METHODS

Materials. Palmitic acid of high purity was supplied by the Hormel Institute. Its melting point was 63 to 64° C. (literature, 63 to 64°). Palmitic acid-1-C^{14} was obtained from the Nuclear Instrument and Chemical Corporation of Chicago. Its specific activity was 2.45 mc. per mM. Counter-current distribution of this material in a 25 tube system, by the method of Ahrens and Craig (10), revealed the presence of 4 to 5 per cent of the radioactivity in a peak of much greater polarity than the main body of the material; this was presumed to represent a short-chain fatty acid impurity. The rest of the radioactivity distributed itself in a single symmetrical peak. The palmitic acid-1-C^{14} was partially purified before use (after analysis) by dissolving it in a 1:1 mixture of isooctane-glacial acetic acid, adding one-tenth volume water to achieve separation into two phases, and discarding the acetic acid phase. This procedure removes most of the short-chain fatty acids from a mixture of short- and long-chain fatty acids (11).

Solutions of sodium palmitate (unlabeled) and sodium palmitate-1-C^{14} were prepared as previously described (12). The concentration of the unlabeled sodium palmitate solution was determined gravimetrically and verified by titration (12); other solutions were made from the initial one by dilution. The concentration of the sodium palmitate-1-C^{14} solution was determined from its concentration of radioactivity and specific activity, using the calibration standards of this laboratory.

The UFA-poor human serum albumin used in these experiments was identical with a previously described preparation (13). This albumin was a sample of Fraction V of Cohn, the long-chain fatty acid content of which had been reduced to 0.1 M per M or less by extracting the lyophilized protein with 5 per cent glacial acetic acid in isooctane. Albumin solutions were freshly prepared for each experiment by dissolving some of the lyophilized protein (stored at −10°) in distilled water. The concentration of each solution was determined by comparing its optical density at 279 mμ as read in a Beckman quartz

spectrophotometer to the optical density of a standard albumin solution whose concentration had been determined by Kjeldahl analysis for nitrogen. Sixty-nine thousand was taken as the molecular weight of albumin for the calculation of molar concentrations.

Slightly outdated normal whole blood which had been collected in acid-citrate-dextrose (ACD) solution was obtained from the Blood Bank of the Clinical Center. After removal of the plasma, the red cells were twice washed by suspension in a solution of phosphate buffer, pH 7.45, ionic strength 0.160 (0.061 M), which was also 0.077 M in NaCl. This solution of phosphate buffer-NaCl is isotonic with respect to plasma. The concentration of red cells in the final preparation (in units of millions of red cells per cu. mm.) was determined by counting the red cells in two chambers of a conventional hemacytometer.

Red cell ghosts were prepared by dilution hemolysis according to the method of Ponder (14). The final preparation of ghosts was suspended in a total volume equal to the volume of packed red cells used in its preparation. An estimated loss of approximately 30 per cent occurred during the process of preparing the ghosts. The concentration of ghosts in the final preparation was hence taken as seven-tenths times the initial concentration of red cells. This estimate is in no way critical in the calculations.

Phosphate buffer was prepared as a stock solution of 10 times the desired ionic strength; dilution of this solution 1 in 10 resulted in a solution of pH 7.45 and ionic strength 0.160.

Method. The experimental technique employed consisted in the equilibration of increasing quantities of sodium palmitate in a mixture of equal volumes of the preparation of red cells (or ghosts) and a solution of serum albumin in isotonic phosphate buffer-NaCl. Equilibration was conducted in a series of ground glass stoppered 50 ml. centrifuge tubes. To each tube were added 2 ml. of the serum albumin solution, 1 ml. of the stock concentrated phosphate buffer, 1 ml. of a solution of 0.770 M NaCl, 1 ml. of the sodium palmitate-1-C^{14} solution, 0 to 5 ml. of an unlabeled sodium palmitate solution, and enough distilled water to make a total volume of 10 ml. The final concentration of serum albumin was slightly greater than 1×10^{-4} M per L. The total quantity of fatty acid in each series of tubes ranged over more than one order of magnitude. To each tube 10 ml. of the red cell (or ghost) preparation was then added, and the tubes equilibrated by being gently tilted back and forth, at the rate of 30 times per minute, for one hour. It was found that equilibration was more than 95 per cent complete at one hour, as compared to six hours. The tubes were then centrifuged for 20 minutes at 1,500 rpm and the clear albumin solutions carefully drawn off. The packed red cells (or ghosts) were washed three times by suspension in 10 ml. of isotonic phosphate buffer-NaCl solution, followed by centrifugation. All experiments were conducted in an air conditioned laboratory at 23 ± 1° C.

After equilibration, the albumin solutions and the washed red cells were analyzed as follows. In the experiments with intact red cells, measured aliquots of each albumin solution and each red cell phase were lyophilized, and extracted with 25 ml. of a 1:1 mixture of isooctane-glacial acetic acid. The mixtures were then split into two phases and the isooctane phases washed according to the method of Gordon (3). Aliquots of the isooctane phases (containing all the palmitic acid) were measured into small vials and evaporated to dryness. In the experiments with ghosts, aliquots of the albumin solutions and the ghosts were extracted with 25 volumes of a solution of ethanol: acetone (1:1), and portions of the extraction solutions measured into vials and evaporated to dryness.[1] To each vial (in both the red cell and ghost experiments) was then added 15 ml. of a scintillation solution consisting of 400 mg. per cent diphenyloxazole (DPO, Pilot Chemicals Inc.) in toluene. The concentration of radioactivity in each vial was measured with a Packard Liquid Scintillation Spectrometer (efficiency, *ca.* 60 per cent; background, *ca.* 15 cpm). Enough counts were recorded so that the standard error of each measurement was 1 per cent or less. From these measurements the total number of cpm associated with each albumin solution and each red cell phase was calculated. The total recovery of cpm was within 5 per cent of the expected recovery in each tube. The total number of moles of palmitate associated with each albumin solution and each red cell phase was then calculated as the per cent of recovered cpm times the known amount of palmitate added to each tube. The average number of moles of palmitate bound per 10^6 red cells was calculated by dividing the number of moles of palmitate found in each red cell phase by the measured number of red cells in millions. Finally, the average number of palmitate ions bound per albumin molecule, $\bar{\nu}$, was determined by dividing the number of moles of palmitate in each albumin solution by the number of moles of albumin present. This calculation assumes that all the palmitate in the albumin solution was bound to the protein; the results of a previous study (8) indicate that the error in this assumption is less than 1 per cent at all concentrations herein employed.

RESULTS

A. *Intact red cells*

The results of the experiments with intact red cells are presented in Table I and are shown graphically as the solid circles in Figure 1, in which $\bar{\nu}$, the average number of moles of palmitate bound per mole of serum albumin, is plotted against the number of moles of palmitate bound per 10^6 red cells. Inspection of Table I reveals that in the concentrations employed a significant fraction of the palmitate was bound by the red cells. The fraction of palmitate bound by the

[1] The more laborious extraction procedure was employed in the experiments with red cells in order to be able to titrate the UFA concentration of the albumin solutions (*vide infra*). This was not done in the experiments with ghosts.

TABLE I

Binding of palmitate by albumin and erythrocytes in vitro

Tube no.*	% of total cpm with RBC's	Total palmitate added	Palmitate in each phase at equilibrium		$\bar{\nu}$ to HSA	Palmitate bound to RBC's	Measured total UFA in HSA phase
			HSA	RBC			
		μM	μM calculated			$M/10^6$ RBC	μM
1	5.79	0.82	0.77	0.047	0.68	0.049×10^{-11}	0.5
2	9.45	1.41	1.28	0.13	1.13	0.14×10^{-11}	1.1
3	14.8	2.58	2.20	0.38	1.96	0.40×10^{-11}	1.7
4	21.1	3.75	2.96	0.79	2.61	0.83×10^{-11}	2.5
5	22.3	4.72	3.67	1.05	3.24	1.10×10^{-11}	3.4
6	27.6	5.70	4.12	1.58	3.64	1.66×10^{-11}	3.6
7	32.7	7.65	5.15	2.50	4.54	2.62×10^{-11}	5.2
8	36.1	10.6	6.75	3.82	5.96	4.00×10^{-11}	6.1

* Each tube contained 1.13×10^{-6} M of human serum albumin (HSA) and 9.54×10^{10} red blood cells (RBC).

red cells is small at values of $\bar{\nu}$ below 2, but increases steadily as the total amount of palmitate present increases.

Calculation of the number of binding sites and their association constants. Scatchard and coworkers have shown (15–17) that the binding of an anion, A, to several classes of sites on a protein molecule may be formulated as:

$$\bar{\nu}_A = \sum_i \bar{\nu}_{Ai} = \sum_i \frac{n_i k'_{Ai} c_A}{1 + k'_{Ai} c_A} \qquad 1)$$

in which k'_{Ai} is the apparent association constant for each of the n_i sites in class i under any particular set of conditions, and c_A is the free (unbound) concentration of A in equilibrium with the protein. The apparent association constant, k'_{Ai}, is valid only for the particular experimental conditions employed (ionic strength, pH, temperature, and so forth), and is related to the intrinsic association constant, k''_{Ai}, by a number of factors which correct for the effects of electrostatic interactions, competition with other ions, and the hydrogen ion equilibrium of the binding sites. These factors have been discussed in detail elsewhere (8).

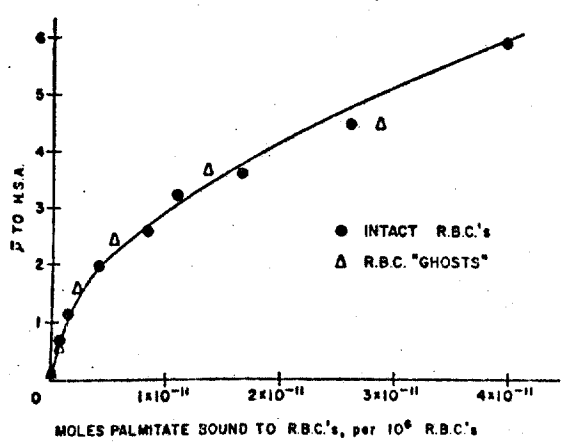

FIG. 1. RELATIONSHIP OF PALMITATE BINDING BY ERYTHROCYTES TO BINDING BY SERUM ALBUMIN

In a recent publication, quantitative studies of the interaction of human serum albumin with several long-chain fatty acid anions were reported (8). The fatty acids studied included lauric, myristic, palmitic, stearic, oleic and linoleic acids. The conditions employed (pH 7.45, 23° C., phosphate buffer, ionic strength 0.160) were identical with the conditions used in the present studies, except for the additional presence of NaCl in the present experiments (to make the solutions isotonic; ionic strength 0.24).[2] Analysis of the data in terms of Equation *1* revealed that in every case there were three classes of sites which bound the fatty acid anions on the serum albumin molecule. The number of sites in each class were: $n_1 = 2$, $n_2 = 5$, $n_3 = 20$. There are therefore a total of 27 sites on the serum albumin molecule which are able to bind fatty acid anions. Of these, two sites bind fatty acids extremely tightly, five sites somewhat less tightly, and the remaining binding sites are relatively weak.

In the same study, the values of the association constants for each class of sites were reported for each fatty acid studied. The values of these constants for palmitate are: $k_1' = 6.0 \times 10^7$;

[2] The addition of this much NaCl has very little effect on palmitate binding to serum albumin (unpublished observations) and has been neglected in the subsequent analysis.

$k_2' = 3.0 \times 10^6$; $k_3' = 1 \times 10^3$.[3] Substituting these values of k′ and the above values of n into Equation *1* therefore yields the following expression for palmitate binding to serum albumin:

$$\bar{\nu} = \frac{1.2 \times 10^8 c}{1 + 6 \times 10^7 c} + \frac{1.5 \times 10^7 c}{1 + 3 \times 10^6 c} + \frac{2 \times 10^4 c}{1 + 10^3 c}. \quad 2)$$

Let us now consider the present experiments, in which the distribution of palmitate between red cells and serum albumin has been measured at a number of different values of $\bar{\nu}$. From these data one can determine the number of sites on red cells which are able to bind palmitate ions, and the tightness of this binding (*i.e.*, the association constants). This was done as follows:

It was first assumed that there is a single class of sites on the red cells which is able to bind palmitate ions; this assumption will be validated below. If we then let R be the number of moles of binding sites per 10^6 red cells, and k_r' be the apparent association constant for each site, the application of Equation *1*[4] yields the binding equation:

$$\bar{y} = \frac{R k_r' c}{1 + k_r' c} \quad 3)$$

in which $\bar{y}$ is the average number of moles of palmitate bound per 10^6 red cells. Solving for c in Equation *3* and substituting this expression for c into Equation *2* then yields an equation relating the experimentally measured quantities, $\bar{\nu}$ and $\bar{y}$, to the two unknowns, R and k_r',

$$\bar{\nu} = \frac{1.2 \times 10^8 \bar{y}}{R k_r' + \bar{y}(6 \times 10^7 - k_r')} + \frac{1.5 \times 10^7 \bar{y}}{R k_r' + \bar{y}(3 \times 10^6 - k_r')} + \frac{2 \times 10^4 \bar{y}}{R k_r' + \bar{y}(10^3 - k_r')}. \quad 4)$$

By the method of successive approximations, the values of R and k_r' which would best fit the experimental data were then determined. These values are: $R = 5.3 \times 10^{-11}$; $k_r' = 2.2 \times 10^6$. The closeness with which these values describe the experimental data can be seen by inspection of Figure 1. The solid curve drawn in this figure is a plot of Equation *4*, using these values of R and k_r'. The fact that such a good fit can be obtained with a plot of Equation *4* validates the initial assumption that there is a single class of binding sites on the red cells. If there were two or more different classes of sites it would be impossible to find values of R and k_r' which would result in a plot of Equation *4* which would give nearly this close a fit. In addition, it should be pointed out that the distribution of the experimental data is such that the solution of Equation *4* for R and k_r' is almost unique.

B. *Red cell ghosts*

The results of the experiments using red cell ghosts instead of intact red cells are also plotted in Figure 1, as open triangles. The results obtained with the ghosts are very similar to those obtained with intact red cells. It is hence apparent that the ability of red cells to bind palmitate ions is a property of the ghosts, and that the binding sites discussed above must be located in the ghosts.

C. *Effect of inhibition of red blood cell metabolism*

In order to treat red cell binding of palmitate ions as a simple chemical equilibrium reaction, it is necessary to demonstrate that this binding is not dependent upon the metabolic activity of the cell, but rather only requires the intact configuration of the binding sites. This was done by preparing two duplicate tubes (with intact red cells) and adding to one of them KCN, to give a final concentration of 0.02 M, and 2-deoxy-D-glucose (Aldrich Chemical Corp.) to give a final concentration of 30 mg. per ml. This quantity of 2-deoxy-D-glucose is enough to inhibit the glycolysis of whole blood by more than 90 per cent (18). The distribution of palmitate between serum albumin and red cells was then measured as described above. The binding of palmitate by the red cells was found to be identical in the two tubes, with 38.1 per cent of the palmitate bound to the red cells in the tube without the CN^- and deoxy-glucose, and 38.6 per cent bound to the red cells in the tube with these additions.

[3] As discussed in Reference 8, the values of n_2 and k_2' are known with less precision than those of n_1 and k_1', and the values of n_3 and k_3' are to be regarded as only little more than approximations.

[4] Equation *1* is just a special form of the mass action expression and is equally valid for red cells as for proteins, so long as equilibrium exists between bound and free small (palmitate, here) ions and units are properly chosen.

D. Chemical analysis of the bound palmitate

Analyses were performed to demonstrate that the labeled palmitate extracted from the red cells after equilibration was still present as an unesterified fatty acid. Aliquots of several of the isooctane extracts of the red cells were combined, and the phospholipids adsorbed onto a silicic acid column (19).[5] The remainder of the extract was separated into neutral lipid and unesterified fatty acid fractions by using the solvent partition method of Borgstrom (20). The phospholipids were eluted from the silicic acid column with absolute methanol. The three fractions (phospholipids, neutral lipids and unesterified fatty acids) were then separately analyzed for radioactivity. The total recovery of radioactivity in the three fractions was 95 per cent. Of this, only 1.2 per cent was found in the neutral lipid fraction, and 2.5 per cent in the phospholipid fraction. These figures are within the limits of error of the techniques employed, and demonstrate that essentially all the labeled material recovered from the red cells was still present as unesterified fatty acid.

An experiment was also performed to rule out the possibility that some of the palmitate found in the washed red cells was still bound to albumin. An equilibration tube was prepared in the usual manner, and to it was added 0.3 μc. of I^{131}-labeled albumin (RISA®, Abbott). After equilibration the red cells were analyzed for both C^{14} and I^{131} activity. I^{131} activity was measured in a well-type scintillation counter. It was found that whereas 34.1 per cent of the C^{14} activity was present in the red cells, only 0.3 per cent of the I^{131} activity was present in the red cells. This indicated that a negligible quantity of albumin was present in the washed red cells, and that the palmitate found there was indeed bound to the red cells.

E. Further measurements

A final check of the validity of the formulations described above was undertaken by titrating aliquots of the isooctane extracts of the albumin solutions for UFA content, using the method of Gordon (3). The results of these titrations were then converted to total micromoles of UFA present in each albumin solution, and are listed in the last column of Table I. If the formulations herein employed are correct, these values should correspond to the palmitate content as calculated from the distribution of radioactivity. These latter values are listed in the fourth column of Table I. It can be seen that the agreement is fairly good, with the measured values varying between 75 and 100 per cent of the calculated values. Most of the variation probably resulted from the experimental error of the UFA titrations, which was ± 0.4 μM UFA per solution in these experiments. These results clearly eliminate the possibility of some gross error being involved in the analytic formulations, such as would occur if the labeled palmitate found in the red cells resulted from an exchange reaction between the palmitate of the solution and some acidic component of the red cells. The treatment of the data in terms of a distribution of palmitate between the binding sites of albumin and red cells is therefore validated.

DISCUSSION

The experimental results presented above demonstrate that human erythrocytes interact strongly with large numbers of palmitate ions, and that this interaction can be described by a mass action expression. It has also been shown that the binding sites are located on the red cell ghost, and that the binding reaction is independent of the metabolic activity of the cell. Analysis in terms of a single class of binding sites has revealed that there are 5.3×10^{-11} M of binding sites per 10^6 red cells, and that the apparent association constant for each of these sites is 2.2×10^6. This number of moles of binding sites per 10^6 red cells can be converted to an equivalent value of 3.2×10^7 binding sites per single red cell, by multiplying by Avogadro's Number and dividing by 10^6. Furthermore, if we divide this number by the average surface area of a normal red cell, 135 μ^2 (21), we arrive at a value for the surface density of binding sites, namely that there is one binding site per 420 square angstroms of red cell surface. These figures show that red blood cells possess an extremely large capacity for binding palmitate ions.

The value of the association constant for the

[5] This was kindly performed by Dr. D. Frederickson and Mr. K. Ono.

red cell binding sites is very close to that of the second class of binding sites on serum albumin (3.0×10^6). The red cell sites can hence compete with the second class of albumin binding sites on an almost equal basis. The association constant for the first class of albumin binding sites is, however, more than an order of magnitude greater than this. The red cells cannot therefore effectively compete with serum albumin until the first class of albumin binding sites is saturated (*i.e.*, until $\bar{\nu}$ is 2 or more). In normal human plasma the concentrations of both serum albumin and unesterified fatty acids are close to 5×10^{-4} M per L. This corresponds to a value of $\bar{\nu}$ of 1. If we assume that all the unesterified fatty acids behave similarly to palmitate, we find, from Figure 1, that the corresponding number of moles of UFA bound per 10^6 red cells is 0.13×10^{-11}. Multiplying by the normal level of red cells in whole blood then indicates that normally only 1 to 2 per cent of the UFA will exist bound to red blood cells.

The demonstration of a high density of potent binding sites on the red cell ghost provides experimental support for the hypothesis that the initial step in plasma UFA metabolism involves the transfer of UFA from serum albumin to receptor sites on cell surfaces. In order for this hypothesis to be correct it is only necessary that there be a large number of rapidly turning over binding sites with association constants within one or two orders of magnitude of the albumin association constants. Under physiologic *in vivo* conditions a true chemical equilibrium is probably never achieved between the binding sites on serum albumin and on cell surfaces, because fatty acids bound to the cell surfaces are probably rapidly transferred inside the cells. The turnover rate of these binding sites is hence extremely important and may, in fact, be the limiting factor in plasma UFA turnover. Nothing is known about the turnover rate of these binding sites, or about the subsequent steps whereby the fatty acids are brought within the cells and to mitochondrial loci of oxidation. These later steps may involve enzymatic processes such as activation, but there are other equally feasible mechanisms which might be postulated.

A similar concept of fatty acid uptake by tissues has been advanced by Fillerup, Migliore and Mead (22), who measured the absorption of palmitate-1-C^{14} by ascites tumor cells in the presence of serum albumin. They found that in the presence of enough KCN to inhibit the respiration of the cells, a large percentage of the labeled palmitate was found associated with the cells. This indicated that the cells could absorb palmitate from an albumin solution in the absence of cell respiration. They suggested that this process of absorption is one of passive partition between the medium and the cell surface. Their experiments did not differentiate, however, between surface and internal binding of palmitate on the part of the tumor cells.

It is true, of course, that red blood cells are atypical cells, and that results obtained with red cells cannot be unreservedly applied to other cells. Red cells were selected because of the ease with which they can be studied in a truly quantitative fashion, and because of the fact that the surface membrane and its associated components (the ghost) can be obtained separately. If one assumes, however, that other tissue cells have a density of binding sites and association constants at all approaching the values found for red cells, the reasonableness of the above hypothesis becomes apparent. The total surface area of tissue cells adjacent to capillaries is much larger than the total red blood cell surface area. It is thus easy to visualize how a large fraction of the plasma UFA could be removed by surface binding sites of tissues, in a single circulation through the tissues. If the fatty acids are able to be transferred inside the cells with great rapidity, the observed very rapid turnover times become understandable. In any event, further studies of this kind using other tissues should provide insight into the details of the mechanisms of unesterified fatty acid metabolism.

SUMMARY

The interaction of human erythrocytes with sodium palmitate has been studied by measuring the distribution of varying quantities of palmitate between human erythrocytes and human serum albumin. The conditions employed were isotonic phosphate buffer-NaCl, pH 7.45, ionic strength 0.24, 23° C. Analysis of the results in terms of a single class of binding sites on the erythrocytes has revealed that there are 5.3 ×

10^{-11} M of binding sites per 10^6 erythrocytes, and that the apparent association constant for each of these sites is 2.2×10^6. This number of binding sites is equivalent to 3.2×10^7 binding sites per erythrocyte. It has also been shown that the binding sites are located on the erythrocyte ghosts, and that the binding reaction is independent of the metabolic activity of the cell. The results have been discussed in terms of the hypothesis that the initial step in plasma unesterified fatty acid metabolism involves the transfer of unesterified fatty acids from serum albumin to receptor binding sites on cell surfaces.

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Hyperlipemia and Hemolysis I. Interaction of Sodium Oleate with Human Erythrocytes. (24851)

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The lytic action of fatty acids on washed mammalian erythrocytes suspended in isotonic saline has long been recognized, although the precise mechanism remains speculative(1,2). Lysis of the same erythrocytes, when suspended in homologous plasma, requires significantly higher concentrations of fatty acid since normal plasma contains potent inhibitors of fatty acid hemolytic activity(3-6). Despite this marked hemolysis-inhibitory property of plasma *in vitro*, evidence has accumulated that *in vivo* hemolysis

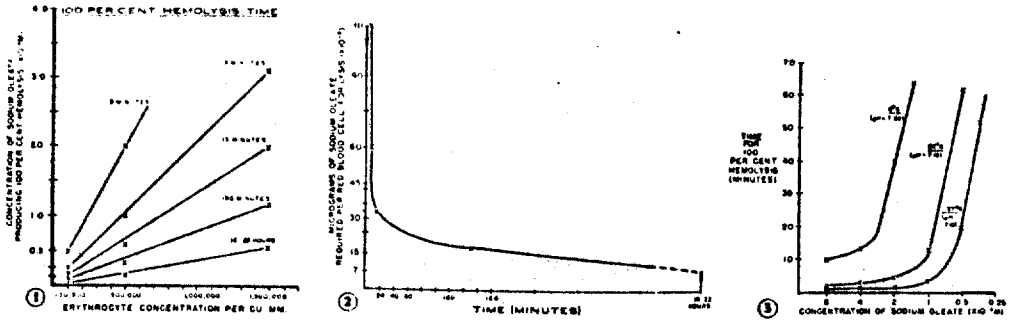

FIG. 1. Relation of human erythrocyte concentration to concentration of oleate required to induce 100% hemolysis at comparable rates at 37°C, pH 7.1.

FIG. 2. Relation of quantity of sodium oleate available per human erythrocyte (in 1.0 ml vol) to 100% hemolysis time at 37°C, pH 7.1. (Data derived from Fig. 1.)

FIG. 3. Effect of temperature on 100% hemolysis time of human erythrocytes exposed to varying concentrations of oleate. Each value represents mean of 5 determinations.

occurs during conditions associated with rapid increases in serum "free" fatty acids.* Thus *in vivo* hemolysis has been reported following alimentary hyperlipemia(8), intravenous infusions of hyperlipemic plasma or lipid emulsions(9,10), or injection of heparin into lipemic animals(11). The mechanisms underlying such *in vivo* hemolysis have not been entirely defined. The present report is the initial observation which may clarify certain intermediate hemolytic pathways.

Methods and materials. Blood of all major groups was collected from normal subjects utilizing citrate or heparin and the erythrocytes separated after 15 minutes at 2500 rpm, 0°C. Red blood cells were washed 3 times in sodium phosphate buffered saline, pH 7.15, ionic strength 0.15. The final erythrocyte suspension was adjusted by Klett-Summerson photoelectric colorimeter (filter 550 mu) so that addition of 0.5 ml of the standardized erythrocyte suspension to 2.5 ml of solution resulted in final concentration of 5×10^5 cells cu mm. Sodium oleate (Baker) was employed as fatty acid: all solutions were prepared in phosphate buffered saline. The effect of temperature on pH of buffered oleate solutions as measured with glass electrode was as follows: 37°C, pH = 7.10; 25°C, pH = 7.15; 0°C, pH = 7.30. With certain specified exceptions, 2.5 ml volumes of fatty acid solutions (or saline controls) were utilized throughout, to which 0.5 ml aliquots of standardized washed erythrocyte suspension were added. All fatty acid solutions remained at room temperature 24 hours prior to use since hemolytic potency declined upon standing, rapidly at first, then more slowly. This phenomenon has been noted previously(12). Surface tension of fatty acid solutions was estimated by measuring rise of fluid into clean glass capillary tubes of 1 mm internal diameter; reproducibility was obtained within ± 4.2%. When this method was checked against reported measurements of temperature effect on surface tension of distilled water (13), the values agreed within ± 5%.

Results. The data presented in each experiment, summarized in Figures and Tables, are representative of similar experiments performed on human erythrocytes obtained from 5 normal subjects:

Effect of erythrocyte concentration on rate of oleate hemolysis. The time required for 100% lysis of human erythrocytes by varying concentrations of sodium oleate at 37°C, pH 7.1, varied with concentration of erythrocytes. Dervichian(14) reported similar results for horse erythrocytes suspended in lauric acid. For any fixed time interval within the ranges tested, concentration of sodium oleate necessary for 100% hemolysis bears a linear relation to concentration of erythrocytes to be lysed (Fig. 1). This finding indicates a stoichiometric interaction of oleate with hu-

* Since present evidence indicates that "free" fatty acids are not free but bound reversibly by serum proteins, the term unesterified fatty acid is preferable(7).

man erythrocytes and suggests that for 100% hemolysis at any given time interval at 37°C, pH 7.1, each erythrocyte binds (or inactivates) a fixed quantity of fatty acid.

Effect of "absorption" of oleate solutions with human erythrocytes. Studies to test the binding of oleate by human erythrocytes were performed by assaying ability of oleate solutions to lyse fresh human erythrocytes after "absorption" with aliquots from the same erythrocyte suspension. Typical results are indicated in Table I. Prolongation in hemolysis time of the supernatant solution obtained after oleate-erythrocyte contact indicates that human erythrocytes bind (or inactivate) oleate; greater binding occurs at 37°C than at 0°C and after increased contact time. The paradoxical increase in hemolytic activity of the supernatant oleate solution when contact is prolonged so that appreciable lysis of the "absorbing" erythrocytes occurs, suggests that following rupture of erythrocyte, hemolytic substances are released.

Evidence for release of hemolytic factors following oleate hemolysis. Standardized suspensions of human erythrocytes were exposed to varying concentrations of sodium oleate at 0°C, pH 7.3 for 30 seconds and spun down

TABLE I. Binding of Sodium Oleate by Human Erythrocytes.

2.5 ml oleate solutions (2×10^{-4} M) "absorbed" with 0.5 ml standardized human erythrocyte suspensions at: Temp., °C	Time*	Optical density of "absorbed" oleate solutions (550 mμ)	100% lysis time of 0.2 ml fresh standardized human erythrocyte suspensions added to 1 ml "absorbed" oleate solutions at 37°C† (min.)
0	0	0	7
"	1	"	10
37	0	20	45
"	.5	32	60
"	1	100	12
"	1.5	400 (100% lysis)	5
Unabsorbed control oleate solution		0	2.5

* Minutes prior to centrifugation at 3000 rpm, 0°C, 3 min.

† Mean of 4 determinations. (The longer the lysis time, the less hemolytically active oleate present. This relation is indicated in Fig. 1.)

TABLE II. Release of Hemolytic Substances after Oleate Lysis of Human Erythrocytes.

0.5 ml standardized human erythrocyte suspensions added to 2.5 ml sodium oleate of conc.:*	100% lysis time of washed red blood cells upon warming to 37°C†	100% lysis time of 0.05 ml fresh standardized human erythrocyte suspensions added to 2.5 ml of:† Saline washing # 2	3	4	Lysed erythrocytes‡
($\times 10^{-4}$ M)	(min.)	(min.)			
8	2.0	>120	>120	>120	1.75
4	"	"	"	"	2.5
2	4.5	"	"	"	7.0
1	50	"	"	"	80

* Erythrocytes added to oleate solutions at 0°C, pH 7.3; after 30 sec., red cells spun down at 3000 rpm, 0°C, for 3 min., then washed 4 times with 3.0 ml buffered saline (pH 7.3) at 0°C and resuspended in a final volume of 3.0 ml buffered isotonic saline.

† Mean of 4 determinations.

‡ All tubes adjusted to same starting optical density with buffered isotonic saline to account for erythrocytes lost during washings.

within 3 minutes at 3000 rpm, 0°C. The oleate supernates were set aside for assay of residual hemolytic activity, and erythrocytes washed 4 times with 3 ml aliquots of iced buffered saline. Despite such washing, 100% hemolysis ensued upon warming to 37°C, the observed rates, (Table II) correlating with those predicted from erythrocyte binding of oleate computed from residual hemolytic activity of the oleate supernates. Tests of the last 3 iced saline washings for lytic activity at 37°C were essentially negative; the final hemolyzed supernate, however, consistently lysed additional erythrocytes. Lysis rate was significantly below that of original oleate solution and was dependent upon initial oleate concentration (Table II). These findings suggest that 1) oleate binding by human erythrocytes is not dissociated appreciably by repeated iced saline washings; 2) erythrocyte binding of oleate in fixed volume increases as the initial oleate concentration is increased; and 3) following oleate hemolysis, hemolytic substances are released. Although it seemed likely that the released lytic material was oleate or products of oleate-erythrocyte interaction, it was necessary to determine whether untreated human erythrocytes contain hemolytic factors or substances that intensify hemolytic activity of oleate traces not removed

TABLE III. Effect of Lysed Human Erythrocyte Constituents on Oleate Hemolysis.

.5 ml standardized human erythrocyte suspensions added to 2.5 ml sodium oleate (pH 7.1, 37°C) containing:		100% hemolysis time (min.)† Oleate concentration ($\times 10^{-4}$ M)		
Lysate* (ml)	Isotonic saline (ml)	4	2	1
	.50	2.5	3.0	5.5
.05	.45	"	"	13
.10	.40	"	5.5	"
.20	.30	"	"	"
.50		"	6.2	30 ‡

* Lysate prepared by repeated freezing and thawing of standardized human erythrocyte suspensions followed by renewal of stroma at 4500 rpm, 0°C, 2 hr. Lysate *per se* was non-hemolytic.

† Mean of 4 determinations.

‡ When 0.50 ml lysate was added 20 sec. *after* suspension of human erythrocytes in the oleate, hemolysis time was 5.5 min.

by washing. Standardized suspensions of human erythrocytes in buffered saline were therefore lysed by repeated freezing and thawing and the stroma removed by 2 hour centrifugation at 4500 rpm, 0°C. The supernatant lysed erythrocyte constituents exhibited no detectable hemolytic activity. Table III indicates the effect of lysed erythrocyte constituents on lytic activity of sodium oleate. The findings reveal that under the defined conditions, normal erythrocyte constituents prepared by freezing and thawing retard oleate hemolysis when added to fatty acid *prior* to insertion of intact human erythrocytes (retarding effect waning as oleate concentration increases) and do not alter oleate hemolysis rate when added 20 seconds *after* oleate-erythrocyte contact. Thus the hemolytic activity observed after oleate hemolysis is probably not attributable to normal erythrocytic factors.

Quantitation of oleate required for lysis of human erythrocytes. As seen in Fig. 1, to shorten the 100% oleate hemolysis time, disproportionately greater increments of oleate are required for any given increment in erythrocytes. This relation can be expressed in terms of mean quantity of oleate required/erythrocyte for 100% lysis at any given time (Fig. 2). The quantity of sodium oleate required/erythrocyte for 100% lysis at 37°C, pH 7.1, becomes asymptotic with respect to time axis; a mean minimum of approximately 7×10^{9} μg of sodium oleate/erythrocyte/1 ml is required for hemolysis to occur at a rate detectably greater than that of control untreated erythrocytes. Conversely, under the same conditions, when greater than 111×10^{9} μg of sodium oleate are available/erythrocyte/1 ml, lysis time is no longer shortened detectably.

Influence of temperature on oleate hemolysis. Temperature variations markedly alter oleate hemolysis rates (Fig. 3). At least 2 mechanisms appear to be involved. Cooling from 37°C to 0°C not only decreases the rate (and/or amount) of erythrocyte oleate binding as indicated by Table I, but also slows the rate at which oleate induces lysis after erythrocyte binding. Thus when standardized suspensions of human erythrocytes were exposed to sodium oleate at 0°C for periods too brief to permit lysis and then washed 5 times with iced buffered saline, those aliquots warmed to 37°C consistently lysed more rapidly than controls held at 0°C. The role of increase in pH of oleate solutions from 7.1 to 7.3 upon cooling from 37°C to 0°C in retarding erythrocyte binding of oleate and slowing hemolysis after binding was not determined.

Effect of non-specific enzyme inhibitors on oleate hemolysis. To determine whether oleate hemolysis involved an enzymatic reaction, several non-specific enzyme inhibitors were tested. Addition of 0.01 M (final concentration) fluoroacetate, fluoride, or azide failed to alter the rate of oleate hemolysis of human erythrocytes. Sodium cyanide, however, added to 1×10^{-4} M sodium oleate (25°C, pH 7.2) delayed 100% lysis of 5 standardized human erythrocyte suspensions from a mean of 3.5 minutes (control) to: 12 minutes at 5×10^{-2} M, 20 minutes at 5×10^{-3} M, and 6 minutes at 5×10^{4} M of cyanide ion.

Role of surface tension reduction on oleate hemolysis. Addition of sodium oleate to buffered isotonic saline lowered surface tension, reduction at any given temperature increasing progressively with increasing oleate concentration. It seemed possible that oleate hemolysis rates might be a function of such surface tension alterations. Fig. 1, however, indicates that within the ranges of oleate and erythrocyte concentrations tested, the 100%

hemolysis time can be maintained constant despite varying fatty acid concentration (and hence varying surface tensions) by altering quantity of erythrocytes added. This suggests that the lowered surface tension to which erythrocytes are *initially* exposed upon introduction into oleate solutions is not an important determinant of 100% lysis time. That surface tension lowering plays no important role even during the latter phases of oleate hemolysis was suggested by experiments in which standardized human erythrocyte suspensions were added to increasing dilutions of 50 x 10^{-2} mg sodium oleate starting with 2 ml solutions; despite an 8-fold dilution and a progressive rise in surface tension, the 100% lysis time at 37°C, pH 7.1, remained constant.

Discussion. The initial step in lysis of human erythrocytes by oleate at pH 7.1 and with concentrations below 8 x 10^{-4} M appears to involve binding of fatty acid by the red blood cell. This was first suggested by the observation that the quantity of oleate required to induce 100% hemolysis within any given time period maintained a linear relation to number of erythrocytes to be lysed. Subsequent studies indicated that contact of normal washed human erythrocytes of all major blood groups with sodium oleate consistently resulted, prior to any detectable hemolysis, in removal of the oleate from solution. Such removal was not proved by chemical analysis, but was indicated by reduced hemolytic activity of the supernatant oleate solution. The alternative possibility that oleate was rendered hemolytically inert is untenable in view of subsequent observations. Moreover, recent evidence demonstrates that human erythrocytes are capable of binding radioactive sodium oleate-I^{131}(15) and sodium palmitate-1-C^{14}(16). Within concentrations employed, the lowered surface tension of oleate solutions did not influence hemolysis rates.

Once human erythrocytes are exposed to oleate under conditions permitting binding of hemolytic quantities, the union is firmly established; lysis cannot be checked by repeated iced saline washings. Hemolysis now proceeds at pH 7.2 ± 0.1 as a function of the quantity of oleate bound and of temperature. The temperature effect is complex. A decrease from 37°C to 0°C slows oleate hemolysis by at least 2 mechanisms: 1) erythrocyte binding of oleate is slowed (and/or reduced), and 2) lytic action of oleate following binding is retarded. Since the quantity of oleate binding is a determinant of hemolysis rate, it might be expected that once erythrocyte oleate binding capacity is saturated, the 100% hemolysis time (at any given pH and temperature) no longer would decrease as oleate concentration increases. This indeed occurs and is attained with a mean of approximately 111 x 10^{-9} μg (36 x 10^{-17} moles) of sodium oleate/erythrocyte at pH 7.1, 37°C. Although this evidence is not conclusive for erythrocyte saturation at these levels, the values may be compared with the mean of 5.3 x 10^{-17} moles of binding sites/human erythrocyte reported for sodium palmitate-1-C^{14} determined at pH 7.45, 23°C(16). The mean minimum quantity of sodium oleate required/human erythrocyte for lysis at rates detectably faster than control untreated red cells at pH 7.1, 37°C is approximately 7 x 10^{-9} μg.

Ability of cyanide ion to retard oleate hemolysis was of interest. Whether this was related to enzymatic interference is unknown; however other non-specific enzyme inhibitors —fluoroacetate, fluoride, and azide—failed to alter oleate hemolysis rates in final concentrations of 0.01 M.

Following lysis of erythrocytes binding oleate, hemolytic factors were released. Since such factors could not be detected from normal erythrocytes lysed by repeated freezing and thawing, it seems probable that oleate (or some product of oleate-erythrocyte interaction) is liberated following oleate hemolysis. Assuming the released hemolytic material to be oleate, the mechanism of release is unknown. Fatty acid probably enters the red blood cell after initial attachment to the surface membrane(1,16); since red cell ghosts bind fatty acid as avidly as intact cells(16), it is tempting to speculate that the lytic factor released following oleate hemolysis represents that fraction of oleate transferred *into* the erythrocyte. Although the hemolytic activity of such released material was always significantly lower than that of the original

oleate solutions, it is apparent that oleate possesses the potential of inducing a decelerating hemolytic chain reaction under proper dynamic circumstances—*i.e.*, when erythrocytes binding oleate lyse after moving into areas populated by normal erythrocytes.

Summary. 1. The initial step in human erythrocyte lysis by oleate at pH 7.2 ± 0.1 and concentrations below 8 x 10^{-4} M involves erythrocyte-oleate binding; oleate binding by a standard number of erythrocytes in a fixed volume is a function of oleate concentration, temperature, and contact time. 2. Once lytic quantities of oleate are bound, hemolysis proceeds at pH 7.2 ± 0.1 as a function of quantity bound and of temperature. Lysis cannot be checked by repeated iced saline washings. A mean minimum of approximately 7 x 10^{-9} μg of sodium oleate is required erythrocyte at 37°C, pH 7.1 for lysis at rates detectably faster than controls: the sodium oleate binding mechanism appears saturated with a mean of approximately 111 x 10^{-9} μg erythrocyte. 3. Lowered surface tension of oleate solutions does not influence hemolysis rates under conditions tested. 4. Cyanide retards oleate hemolysis; other nonspecific enzyme inhibitors fail to do so. 5. Following oleate hemolysis, hemolytic substances are released; these probably represent oleate or products of oleate-erythrocyte interaction. It is suggested that oleate possesses the potential of inducing a decelerating hemolytic chain reaction.

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Lipogranuloma from Dietary Saturated Fats: Production and Reversal

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Possible significance in human nutrition of lipogranuloma, a foreign-body-type reaction in adipose tissue of animals fed high levels of saturated fat, justified detailed study of its dietary production. Lipogranuloma was discovered in fat cells of rats fed saturated fatty acids as acetylated monoglycerides (Ambrose *et al.*, 1958; Herting and Crain, 1956), but Herting and Crain (1958) found that other saturated fats added to the diet at relatively high levels induced this fat-cell response. Lipogranuloma is characterized by multinucleation and a granular pink-staining material in adipose tissue; the histologic description was published by Cox and DeEds (1958) and Herting and Crain (1958). This communication describes our experiments showing the rapidity with which various saturated fats induce lipogranuloma and also showing that this fat-cell reaction can be reversed easily and completely by replacing the saturated fat in the diet by unsaturated fat. A preliminary report of some of these data was given by Herting *et al.* (1959).

METHODS

Weanling rats (Holtzman) were distributed randomly to form groups of 48 to 72 animals, an equal number of males and females in each group. Animals were housed in groups of four in wire mesh cages. Food and water were given ad libitum.

Effect of Various Saturated Fats

Diets and fats used are described in Tables 1 and 2.

TABLE 1

COMPOSITION OF DIETS TO PRODUCE AND REVERSE LIPOGRANULOMA FORMATION

Ingredients	Fed 24 weeks to produce lipogranuloma: Groups VIII	IX	X	XI	XII	XIII	XIV	XV	Fed 24 weeks to reverse lipogranuloma: Groups VIII-XI: XIII-XV
Casein	20	20	20	20	20	20	20	20	20
B-vitamin casein	4	4	4	4	4	4	4	4	8
Salt mixture, USP XIV	5	5	5	5	5	5	5	5	5
Corn oil (+ fat-soluble vitamins)	4	4	4	4	4	4	4	4	20
Cellulose	—	—	—	—	—	—	5	—	—
Glucose	17	17	17	17	57	17	17	17	47
Acetylated monoglycerides	50	—	—	—	—	—	—	—	—
Hydrogenated lard	—	50	—	—	—	—	—	—	—
Palmitic acid	—	—	50	—	—	—	—	—	—
Lard	—	—	—	50	—	—	—	—	—
Triacetin	—	—	—	—	10	—	—	—	—
Stearic acid	—	—	—	—	—	50	—	—	—
Ethyl stearate	—	—	—	—	—	—	45	—	—
Monoglycerides	—	—	—	—	—	—	—	50	—

TABLE 2

CHEMICAL CHARACTERISTICS OF FATS USED

Group	Dietary fat	Source	Melting point[a] (°C.)	Iodine no.[b]	Free acid content (%)
VIII	Acetylated monoglycerides from hydrogenated lard	DPI MYVACET Type 5-00	39–57	0.8	0.9[c]
IX	Hydrogenated lard	Archer-Daniel Hydrofol T57L	62–63	0.2	0.4
X	Palmitic acid	Armour Neo-fat-16	58–60	0.2	99.2
XI	Lard	Arpeako prime steam-rendered	37–47	66.6	<0.1
XII	Triacetin	Eastman Organic Chemicals	—	—	—
XIII	Stearic acid	Humko Hystrene S-97	66–67	<0.1	99.3
XIV	Ethyl stearate	Mathe Chemical Company	23–25	0.6	0.3
XV	Monoglycerides from hydrogenated lard	DPI MYVEROL Type 18-00	61–73	0.6	1.3
All	Corn oil	Corn Products Mazola	—	125.8	<0.1

[a] Determined according to Official Methods, American Oil Chemists' Society, Section Cc 1-25 (capillary method).
[b] Wij's method, A.O.C.S., Section Cd 1-25.
[c] Maximum value. Calculated on basis that all free acid is long-chain fatty acid.

The control group for this experiment (group XII) was fed a diet containing 4 % corn oil and 10 % triacetin (supplying acetyl equivalent to that in a diet containing 54 % of distilled partially acetylated monoglycerides). Since lipogranuloma was first seen in adipose tissue from rats fed saturated acetylated monoglycerides, this group served the dual purpose of determining whether the acetate group shared responsibility for the lesion and also of indicating whether short-chain acids in general would produce the lesion. This diet is also the closest to a fat-free diet that we have tested.

Reversibility with Unsaturated Fat

After 24 weeks on the above diets, all rats except those fed triacetin (group XII) or distilled monoglycerides (group XV) made from hydrogenated lard were changed to a diet containing 20 % corn oil (Table 1). The rats fed monoglycerides (group XV) were not switched until 40 weeks, and those on triacetin (group XII) were never changed. The rats remaining after 48 weeks were killed in all groups except the one fed monoglycerides. Three of these were saved for 4 additional weeks.

The number of rats killed at each 8-week interval varied according to the number left in each group. They were chosen whenever possible so that the average body weight of those remaining was essentially unchanged. Pieces of fat attached to adrenal, kidney, stomach, testis or ovary, and abdominal wall were fixed with Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Two sections from 10 to 30μ apart were taken from each tissue.

The sections were identified by code number and were correlated with dietary groups after completion of microscopic examination. The sections were first examined with a scanning microscope and the amount of fat attached to the tissue or organ was rated as one, two, or three, according to whether it filled one or less, one to two, or two to three fields, respectively, using a 3 X objective lens with a 10 X eyepiece. A high-powered objective lens was then used to search for the foreign-body reaction in all fields of fat and to count the foci of reaction, which ranged from a single cell to a group of adjoining involved cells. An arbitrary rating of severity was made by dividing the number of foci of reaction by the number of fields of fat. A rating of "slight" meant an average of one or less focus of lipogranuloma per field of fat. "Moderate" meant an average of one to five foci of lipogranuloma per field of fat. "Severe" meant an average of more than five foci of lipogranuloma per field of fat.

RESULTS

Examination of the fat associated with the various tissues showed that the incidence of lipogranuloma was greater in the fat associated with the testis or ovary than with any other tissue. Of the 225 animals showing no incidence in perigonadal fat (Tables 3 and 5), only 14 showed a reaction in any other adipose tissue. Severity of reaction, however, showed no correlation with location of fat in the body. To conserve space, only the data on perigonadal fat are presented. Differences in incidence due to sex were not significant; they were 62 and 57 % in males and females, respectively.

Incidence and severity of the lesion in rats fed various saturated fats are summarized in Table 3. The foreign-body reaction appeared most rapidly in rats fed palmitic acid, stearic acid, ethyl stearate, and acetylated monoglycerides, less rapidly with fully hydrogenated lard, and least rapidly with monoglycerides. However, severity varied greatly within each group.

Absorbability (the percentage of ingested fatty acids absorbed) of the fats in the various diets was determined for a 1-week period during the twelfth and thirteenth weeks of the experiment (Table 4). Six rats were used in each group except group XIII males, in which there were four. Each of the diets contained 4 % of corn oil in addition to the 45 or 50 % level of test fat. Group XII, fed 10 % triacetin plus 4 % corn oil, was used as a control, thus making it unnecessary to correct for the saturated fatty acids supplied by the corn oil (0.4 g saturated fatty acids per 100 g diet). The absorbability of lard (group XI) was 94.7 % for male and 96.8 % for female animals.

Comparison of the absorbabilities of the various saturated test fats with the rapidity of development and severity of lipogranuloma induced by these fats showed an approximate direct relationship.

Following transfer from the diets containing saturated fatty acid to that containing corn oil, lipogranuloma diminished rapidly in both incidence and severity within 8 weeks. It had disappeared completely in most groups by the end of the experiment (Table 5). No signs of permanent scarring or necrosis were observed in the adipose tissue.

DISCUSSION

Nutritional studies in acetylated monoglycerides showed that these fats are handled by the body much as are the triglycerides from which they are derived (Herting *et al.*, 1955; Ambrose and Robbins, 1956; Mattson

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TABLE 3

THE EFFECT OF VARIOUS SATURATED FATS ON INCIDENCE AND SEVERITY OF LIPOGRANULOMA

Group	Fat	Saturated fatty acid (% of diet)	Time (wk)	Rats	Reaction in perigonadal fat: None	Slight	Moderate	Severe
VIII	Acetylated monoglycerides from hydrogenated lard	35.8	8	6	2	0	3	1
			16	6	1	1	2	2
			24	7	0	0	7	0
IX	Hydrogenated lard	48.2	8	7	6	1	0	0
			16	8	5	3	0	0
			24	8	3	2	3	0
X	Palmitic acid	50.4	8	6	3	0	1	1
			16	6	2	0	2	2
			24	5	1	1	2	1
XI	Lard	19.0	8	8	8	0	0	0
			16	8	8	0	0	0
			24	7	7	0	0	0
XII	Triacetin	0.4	8	8	8	0	0	0
			16	8	8	0	0	0
			24	8	8	0	0	0
XIII	Stearic acid	50.4	8	7	2	1	0	4
			16	6	3	0	0	3
			24	5	0	1	3	1
XIV	Ethyl stearate	41.4	8	6	2	2	2	0
			16	5	1	0	2	2
			24	6	1	2	0	3
XV	Monoglycerides from hydrogenated lard	39.8	8	7	7	0	0	0
			16	8	8	0	0	0
			24	7	6	1	0	0
			32	7	5	2	0	0
			40	7	3	3	0	1

TABLE 4

ABSORBABILITY OF SATURATED FATTY ACID FED AS VARIOUS COMPOUNDS

Group	Fat	Sex	Food consumption (g/rat)	Saturated fatty acid balance: Consumed (g)	Excreted (g)	Absorbed (g)	Absorbability (%)
VIII	Acetylated monoglycerides from hydrogenated lard	M	133.3	47.2	24.6	22.6	47.8
		F	115.7	40.9	18.6	22.3	54.5
IX	Hydrogenated lard	M	179.5	85.8	68.9	16.9	19.7
		F	132.7	63.4	51.7	11.7	18.4
X	Palmitic acid	M	85.5	42.8	18.0	24.8	58.0
		F	91.7	45.8	18.4	27.4	59.8
XIII	Stearic acid	M	142.7	71.3	51.3	20.0	28.0
		F	141.2	70.6	55.6	15.0	21.2
XIV	Ethyl stearate	M	139.2	57.0	29.7	27.3	47.9
		F	123.5	50.6	26.1	24.5	48.4
XV	Monoglycerides from hydrogenated lard	M	189.2	74.6	62.4	12.2	16.3
		F	138.5	54.6	42.2	12.4	22.7

et al., 1956). It was anticipated, therefore, that all saturated fatty acid preparations tested in these experiments would produce lipogranuloma when the amount of dietary unsaturated fatty acid was relatively low. This proved to be true; fully hydrogenated lard, palmitic acid, stearic

TABLE 5

REGRESSION OF LIPOGRANULOMA BY FEEDING CORN OIL

Group	Former dietary fat	20% Corn oil (wk)	Rats	Reaction in perigonadal fat			
				None	Slight	Moderate	Severe
VIII	Acetylated monoglycerides from hydrogenated lard	0	7	0	0	7	0
		8	6	3	0	2	1
		16	3	2	1	0	0
		24	5	5	0	0	0
IX	Hydrogenated lard	0	8	3	2	3	0
		8	7	7	0	0	0
		16	6	6	0	0	0
		24	7	7	0	0	0
X	Palmitic acid	0	5	1	1	2	1
		8	5	3	1	0	1
		16	2	2	0	0	0
		24	1	1	0	0	0
XI	Lard	0	7	7	0	0	0
		8	6	6	0	0	0
		16	4	3	1	0	0
		24	4	4	0	0	0
XII	Triacetin	0	8	8	0	0	0
		8	7	7	0	0	0
		16	7	7	0	0	0
		24	7	6	0	0	0
XIII	Stearic acid	0	5	0	1	3	1
		8	4	3	0	0	1
		16	5	2	1	2	0
		24	5	3	2	0	0
XIV	Ethyl stearate	0	6	1	2	0	3
		8	3	2	1	0	0
		16	3	3	0	0	0
		24	3	3	0	0	0
XV	Monoglycerides from hydrogenated lard	0	7	3	3	0	1
		8	6	6	0	0	0
		12	3	3	0	0	0

acid, ethyl stearate, and monoglycerides and acetylated monoglycerides made from hydrogenated lard all produced lipogranuloma. Thus, the tentative conclusion of Ambrose *et al.* (1958) that the foreign-body-type reaction is due to "acetostearin" should be extended in the light of subsequent findings. The causative agent of lipogranuloma is excessive intake of saturated long-chain fatty acid, regardless of the source of this fatty acid.

Acetate fed as triacetin and at a level comparable to that represented by 54 % distilled partially acetylated monoglycerides did not produce the reaction; this is in line with similar findings by Ambrose *et al.* (1958), who fed triacetin and sodium acetate. By analogy, it seems unlikely that lipogranuloma will be caused by other short-chain fatty acids not considered as being "fat forming" (C_8 and less) or perhaps distinguished by absorption primarily via the portal vein instead of the lymph (C_{10} and less) (Deuel, 1955).

Removal of the saturated fat from the diet and addition of corn oil led to rapid changes in the chemical characteristics of the depot fat (increased iodine values, decreased melting point, fewer triglycerides containing saturated fatty acids) and prompt diminution and eventual disappearance of the foreign-body-type reaction. Only two rats showed the reaction after 24 weeks on the diet containing 20 % corn oil (40 % of calories), and the reaction was minimal in these rats. In all rats, the gross and microscopic appearance of the adipose tissue was completely normal following disappearance of the lesion.

The fact that lipogranuloma can be reversed by substituting a diet in which the unsaturated:saturated fatty acid ratio is 8.1 (corn oil) for diets in which the ratio is in the range of 0.07 to 0.10 (saturated fat) (groups VIII–X, XIII–XV) shows that there is a critical ratio of unsaturated: saturated fatty acid intake above which lipogranuloma formation is unlikely. Actually, the critical ratio is less than 1.72, the calculated value for the fat (predominantly lard) fed to group XI, since this diet did not produce lipogranuloma. Experiments establishing the critical ratio will be reported in a subsequent paper. In the meantime, it is interesting to calculate the ratio of unsaturated:saturated fatty acids from the recent survey of the mean per capita consumption of fatty acids (United States Department of Agriculture, 1957) and to find that this ratio is 1.5. (Of the total intake of long-chain fatty acid, 60 % was unsaturated and 40 % was saturated.)

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SUMMARY

Lipogranuloma, a foreign-body-type reaction in adipose tissue, was observed in rats fed diets containing 35–50% levels of saturated fatty acids fed as palmitic acid, stearic acid, ethyl stearate, hydrogenated lard, or monoglycerides or acetylated monoglycerides made from hydrogenated lard. The incidence and severity of the reaction were approximately parallel to the amount of saturated fatty acid absorbed. Control groups fed lard or triacetin did not develop lipogranuloma during the 48-week test period.

Replacing the diets containing saturated fat with one containing 20% corn oil led to prompt diminution (evident in all groups after 8 weeks) and eventual disappearance (essentially absent from all groups after 24 weeks) of the lipogranuloma. After reversal of the reaction, the adipose tissue showed no signs of damage.

ACKNOWLEDGMENTS

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STUDIES ON ABSORPTION OF LEUCOMYCIN FROM INTESTINAL TRACT

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It is indispensable for the drug administered orally to be absorbed from the digestive tract and to diffuse into blood and organs. As reported in the previous paper, leucomycin (LM) is absorbed from the intestinal tract when it is given orally, but the blood level is relatively low and assayable blood level is not maintained for a long time. The present experiments have been undertaken to obtain improved preparations with higher and longer blood levels.

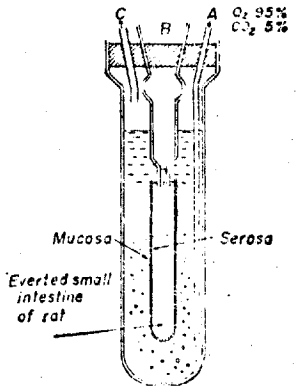
In quantitative examination of LM absorption following its oral administration, a remarkable inactivation was noticed when LM was absorbed from intestinal tract. Therefore, the experiments aimed at discovery of substances which prevent such inactivation of LM, combination of such substances with LM, and evaluation of combined administration to mice. The results are presented in this paper.

Materials and Methods

1. Test method for absorption from the small intestine.

Male rats of Donryu strain weighing about 120 g were raised with solid feed. After they were sacrificed by blood letting, 10 cm long of ileocecal region was cut out by scissors at a site of 15 cm above the caecum, everted on a filter paper with a spatula to remove moisture, ligatured one end, and connected to KRAWN-WILSON's glass tube to form a sac. As shown in Fig. 1, the sac is put in a test tube through the stopper, and accurately measured 1 ml of RINGER's solution is poured into the sac. The KREBS-RINGER's solution of sample warmed at 37°C is poured into the test tube outside of the sac. The outer solution is aerated with oxygen gas containing 5% carbon dioxide through a small glass tube (A). The inner solution is taken out at a given interval (B) to estimate the amount of substance absorbed into the sac of everted rat intestine from outer mucosa surface.[8,9,10] The inner solution comes up into (B) by the pressure of oxygen gas introduced from (A) when (C) is closed with a finger tip.

Fig. 1. Sac apparatus



It is convenient to use this apparatus for a microanalysis of the drug absorbed from the intestine. In the present experiment, LM solution was used as outer solution, and RINGER's solution as inner one.

2. Preparation of intestinal tissue homogenate.

A certain length of rat intestine was taken out, added with KREBS-RINGER's solution twice the weight of wet tissue, and ground using Potters' grinder to obtain the tissue homogenate. LM was added to the homogenate to examine the adsorption of LM on the tissue.

3. Estimation of blood levels.

Groups of *dd* mice, consisting of 3 mice each were administered orally with certain doses of LM alone or in combination with other drugs. The tips of their tails were cut with a sharp razor to obtain blood samples. The blood level was estimated by the paper

disc method dropping a blood sample of each mouse directly on a disc, and the average blood level of 3 mice was calculated. After a required amount of blood was taken, the tail was tied with a suture thread to stop bleeding, and next sampling was done by upper region of the cut tail. Thus, it is possible to obtain several test samples.

Experimental Results

1. Absorption of LM from Intestinal Tract in Sac Apparatus

(1) Absorption of LM

The RINGER's solutions dissolving 3 and 6 mcg/ml of LM were used as the outer solution (mucous fluid). One ml of RINGER's solution was put into the sac. The apparatus was kept in a water bath at 37°C, and the inner solution (serosus fluid) was sampled at intervals. Their activities were estimated by a superposing method using *Sarcina lutea* PCI 1001 as the test organism.

The result is indicated in Fig. 2. LM was found to be absorbed more from the mucous membrane when the LM concentration increased.

(2) Effect of dinitrophenol and pyridoxal-5-phosphate on LM absorption

Three sets of sac apparatus were used. The RINGER-ROCK's solution containing 250 mcg ml of LM was used as the outer solution. On the first set, LM concentration of the inner solution was estimated at a given interval, and served as the control. To the outer solution of the second set, dinitrophenol (DNP) solution was added at 5 minutes after the initiation of the experiment to make the final concentration of DNP 10^{-4} mol. Similarly, 10^{-4} mol of DNP was added to the third apparatus after 5 minutes, and 2 mols of pyridoxal-5-phosphate (PALP) after 12 minutes.

The absorption of LM into each sac was examined at intervals. The result is illustrated in Fig. 3, in which the amount of LM was represented by mcg per 100 mg of dried intestinal wall. The absorption of LM from intestinal tract was not inhibited by DNP, and accordingly no recovery was noticed with PALP.

(3) Effect of DNP and PALP on L-histidine absorption.

As to L-histidine similar experiments were carried out. As shown in Fig. 4, the absorption of L-histidine was inhibited remarkably by DNP, and found to be recovered by the addition of PALP. This fact suggests that LM is absorbed from intestine by a mechanism different from that of L-histidine.

Fig. 2. Amount of leucomycin in the mucous fluid

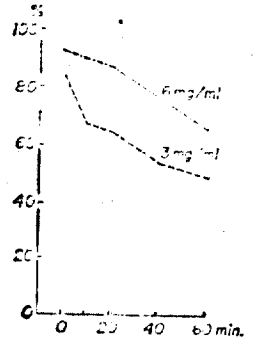
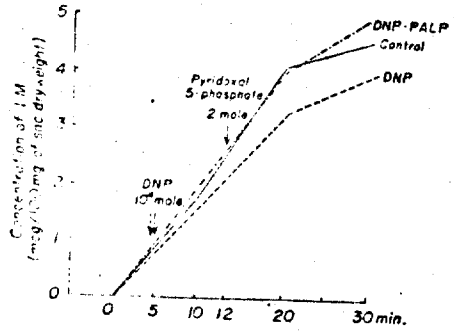


Fig. 3. Amount of LM transported in the sac



(4) Absorption and distribution of LM tested by sac apparatus.

One ml of RINGER's solution was put into the everted sac, and each 10 ml of RINGER's solution containing 30, 300 or 3,000 mcg/ml of LM were used as the outer solution. The apparatus were incubated at 37°C for 40 minutes aerating with oxygen gas containing 5% carbon dioxide to estimate the content of LM contained in the inner and outer solutions.

The LM concentrations in these solutions and intestinal tissue are indicated in Table 1. In the experiment with 300 mcg/ml LM, in spite of the decrease of LM content in the outer solution to 172 mcg/ml (57%) after 40 minutes, only 21.7 mcg/ml (7%) were detected in the inner solution, and 1.9 mcg/ml (0.6%) in the intestinal tissue. Therefore, 104 mcg/ml (35%) in balance were lost. Similar facts were observed in the experiment with 30 and 3,000 mcg/ml of LM. It was found that 23~35% of LM was lost in the process of absorption.

Fig. 4. Amount of L-histidine transported into the sac

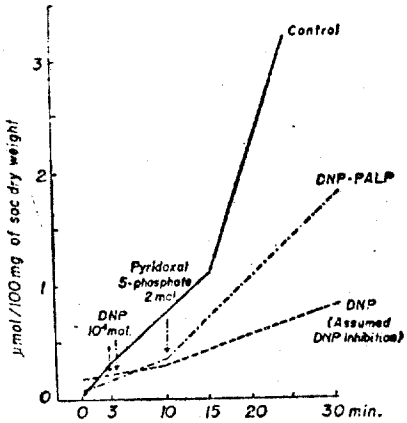


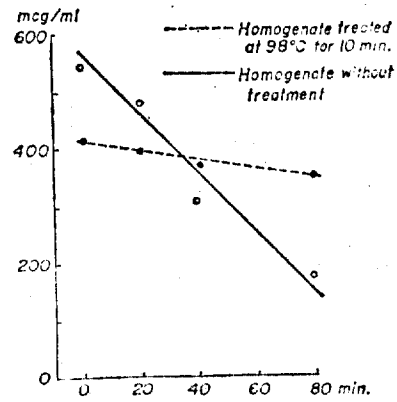
Table 1. Absorption of LM by the sac method.

Experiment No.	Initial concentration, mcg/ml	Final concentration, mcg/ml			Total
	Mucous	Mucous	Serous	in tissue	
I	30	22.6 (75.4%)	0.21 (0.7%)	0.31 (1.3%)	23 (77%)
II	300	172 (57%)	21.7 (7%)	1.9 (0.6%)	196 (65%)
III	3,000	1890 (63%)	202 (7%)	234 (8%)	2326 (77%)

2. Inactivation of LM by Intestine Homogenate

As it was made clear in the above-described experiment that LM lost its activity considerably when it was absorbed from intestinal canal, following experiments were undertaken to examine the cause of inactivation. The intestinal canal of rats was ground in Potter's homogenator, added with twice the weight of LM-KREBS-RINGER's solution to make a final concentration of LM 600 mcg/ml, and incubated at 37°C for 80 minutes. During the incubation, aliquot samples were pipetted to estimate LM concentration in the homogenate. The result is indicated in Fig. 5, in which dotted line represents a control experiment that the intestine homogenate was heated at 98°C for 10 minutes, added with the same amount of LM and incubated similarly.

Fig. 5. Amount of leucomycin in the intestinal homogenate



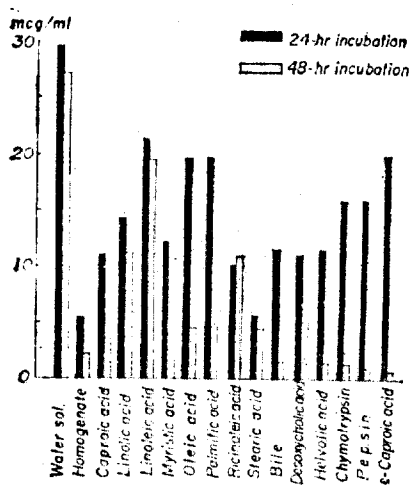
The result indicates that the activity of LM is lost in the rat intestine homogenate at the ratio of 7% after 20 minutes, 40% after 40

minutes, and 74 % after 80 minutes, and that the LM inactivating factor in the intestine homogenate is weakened considerably by heating.

3. Inhibition of LM Inactivation by Intestine Homogenate

As it was proved in the previous experiments that the activity of LM was lowered by the intestine homogenate, a variety of fatty acids, proteolytic enzymes, bile acids and other substances were added to the LM plus intestine homogenate, incubated at 37°C for 24 hours, and estimated for the activity of LM remained.

Fig. 6. Antibacterial activities of LM mixed with various substances.

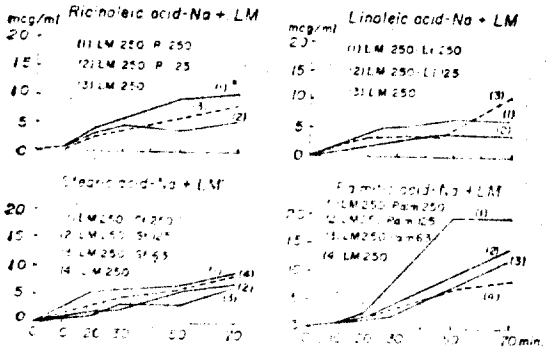


In Fig. 6 are depicted the results of the experiment in which each 100 mcg/ml of a variety of fatty acids was added to 0.5 ml of rat intestine homogenate prepared by adding KREBS-RINGER's solution twice the weight of wet intestine and 1 ml of 50 mcg/ml LM solution, and LM activities were estimated before and after the incubation at 37°C for 24 and 48 hours. It was found that the inactivation of LM in the homogenate was slight with linoleic acid and caproic acid at 24 hours. But the activity became considerably low with the acids, except linoleic acid at 48 hours.

4. Effect of Various Substances on Absorption of LM from Intestinal Canal

To test the combined effect of various substances with LM *in vitro*, the passage of mixtures of various substances and LM through intestinal canal was observed by using WISEMAN's apparatus described above. The final concentration of LM in this experiment was 250 mcg/ml, and the following substances were mixed with LM solutions:

Fig. 7-1. Amount of LM in the serous fluid



(1) Higher fatty acids: Sodium ricinoleate, 250, 125 mμg/ml; sodium linolate, 250, 125 mμg/ml; sodium stearate, 250, 125 mμg/ml.
(2) Sodium ε-caproate, 200, 100 mμg/ml.
(3) Bile powder, 400, 200, 100 mμg/ml.
(4) Chymotrypsin, 200, 100, 50 mμg/ml; Pepsin, 200, 100 mμg/ml.
(5) Sulfa drugs: Sulfisomezole, 50 mμg/ml; sulfathiazole, 50, 10 mμg/ml

The mixtures of LM and above substances were added to the mucous fluid, and LM concentrations in serous fluid were estimated after 10, 20, 30, 50 and 70 minutes. The results are indicated in Fig. 7. The absorption of LM from intestinal canal was not promoted by the addition of sodium linolate and sodium ricino leate which

gave better results in the experiment using homogenate. However, the absorption was promoted by sulfisomezole, sodium palmitate, sodium ε-capronate, chymotripsin and sulfathiazole in order.

5. Effect of Various Substances on Blood Level of LM

To test *in vivo*-combined effect of LM and various substanc s whether *in vivo* experiment run parallel with *in vitro* one, various substances tested *in vitro* were mixed with LM, and administered orally to groups of 3 mice of *dd* strain. The dose of LM was 50 mg per kg of body weight. The LM blood levels were examined by paper disc method from 30 minutes to 10 hours after the administration.

As shown in Fig. 8, the mixtures of higher fatty acids with LM failed to produce higher blood level and to prolong its duration in spite of their favorable results *in vitro*. However, the LM blood level increased about twice as much as the control by combining with sulfisomezole, sulfathiazole, chymotrypsin, deoxycholic acid, bile and the assayable blood level continued for 8~10 hours after the administration.

Discussion

The present paper dealt mainly with the relation of absorption from intestinal canal and blood level of LM.

Fig. 7-2. Amount of LM in the serous fluid

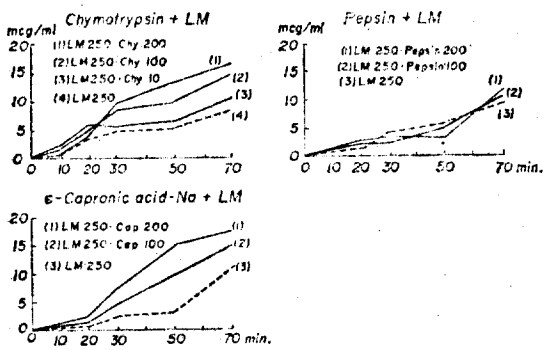
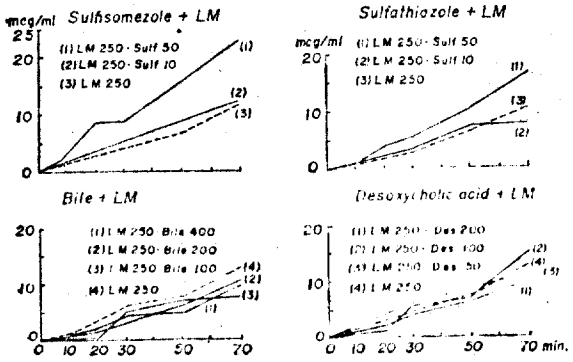


Fig. 7-3. Amount of LM in the serous fluid



The *in vitro* method for the absorption from intestinal canal, using the everted sac of rat intestine estimates the amount of substance which is absorbed from mucous membrane and diffused through serous membrane. Although it is possible to estimate the speed of diffusion from the mucous membrane to the serous one by this method, it is impossible to presume directly the absorption *in vivo*. When LM is put in the solution outside of the everted sac, and RINGER's solution inside of the sac, the concentration of LM in the sac increases with the times.

Generally, in the intestinal canal, the active transport occurs only from mucous surface to serous one, and the diffusion occurs only from serous surface. Therefore, the difference of their speeds causes an increase of LM concentration in the sac with a lapse of the time. According to WISEMAN, it is a specific role for the intestinal canal to absorb rapidly and efficiently the nutriments required in the body. As the diffusion velocities of water-soluble and non-liposoluble substances are very slow, the highly efficient mechanism is required *in vivo*. The sugars and amino acids which are indispensable for a living body are absorbed from the intestinal canal by so-called active transport mechanism. The active transport means the passage of substances through a biological

Fig. 8-1. Blood level of LM in mice after oral administration of the mixture of LM and other agents (mg/kg)

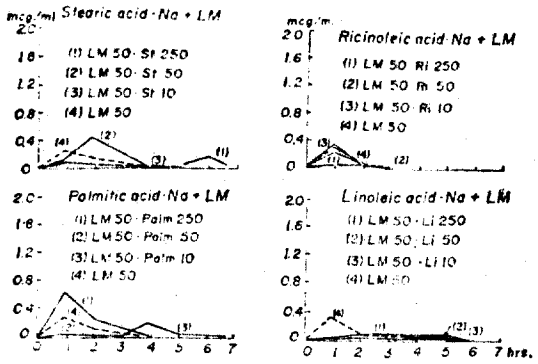


Fig. 8-2. Blood level of LM in mice after oral administration of the mixture of LM and other agents (mg/kg)

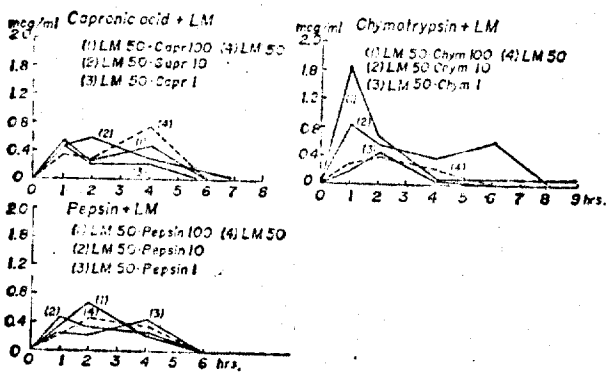
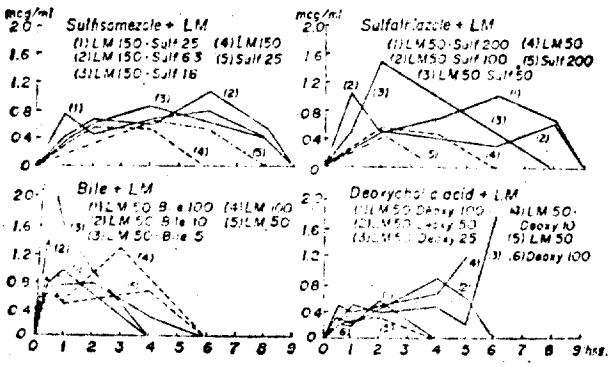


Fig. 8-3. Blood level of LM in mice after oral administration of the mixture of LM and other agents (mg/kg)



membrane counteracting electrochemical gradient, and required the energy generated in the cells. It is a different mechanism from the passive diffusion in whcih substances are absorbed by the difference of concentrations, and from the phagocytosis of protozoa such as amoeba and euglena.

It has been found that the absorption by active transport mechanism is inhibited by the substances inhibiting generation of energy. In the present experiment *in vitro*, the absorption of LM was recognized to be a passive diffusion type, as it was not affected by dinitrophenol (an ATP inhibitor) and pyridoxal phosphate, unlike L-amino acids absorption that involves a reaction coupled with energy and is inhibited or promoted by dinitrophenol or pyridoxal phosphate.

The activity of LM in homogenate of intestinal tissue was found to be inactivated at a ratio of 20 % after 20 minutes, and 74 % after 80 minutes. Although it is unknown how long LM remains in the intestinal tissue of which condition is not conceivable to be the same as the intestinal homogenate, LM is considered to be inactivated to some extent in the normal tissues. The substances of which effect were observed *in vitro*, such as various fatty acids, proteolytic enzymes and sulfa drugs combined with LM were administered orally to mice, but no effect was noticed with higher fatty acids, whereas some effect with sulfa drugs and proteolytic enzymes. The contradiction between *in vivo* and *in vitro* experiments apparently indicates an absorption mechanism in the intestinal canal involves more complicated factors than that in the sac.

Summary

1. The sac method indicated that LM is absorbed from intestinal canal by the passive diffusion, and is not influenced by dinitrophenol and pyridoxal phosphate. On the contrary, L-histidine is

absorbed by the active transport that is affected by dinitrophenol and pyridoxal phosphate.

2. LM is inactivated considerably by the intestinal homogenate.

3. Linoleic acid inhibits the inactivation of LM in the intestinal homogenate, but other higher fatty acids, proteolytic enzymes and bile acid failed to inhibit the inactivation.

4. Sulfa drugs (sulfisomezole, sulfathiazole), sodium palmitate, chymotripsin and sodium ε-caproate increased comparatively the absorption of LM from mucous membrane *in vitro* experiment.

5. The blood level of LM after oral administration of LM was enhanced by the combinations with bile powder, chymotrypsin and dosoxychol acid. The duration of assayable blood level was prolonged with the combination of sulfa drugs (sulfisomezole, sulfathiazole), sodium ε-caproate, chymotrypsin, bile and deoxycholic acid but not with fatty acids.

6. Accordingly, the *in vitro* results almost agreed with the *in vivo* ones with sulfa drugs, ε-caproate and chymotrypsin.

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Specifications for the Identity and Purity of
Some Food Colours, Emulsifiers and Anti-Caking
Agents and Certain Other Substances

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Toxicological Evaluation of Some Food
Colours, Emulsifiers, Stabilizers and Anti-Caking
Agents and Certain Other Substances

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PLATELET-AGGREGATION BY PHOSPHOLIPIDS AND FREE FATTY ACIDS

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THE comparative importance of thrombosis and disordered lipid-metabolism in the production of ischæmic heart-disease has not yet been established. Studies on the pathogenesis of ischæmic heart-disease tend to emphasise one hypothesis at the expense of the other, but it is possible that both mechanisms play a part and may indeed be promoted by a common factor. Epidemiologically there is much to support this view. In Norway, for example, in parallel with the fall in mortality from circulatory diseases during the late war, there was a fall in thromboembolic complications (Strom and Jenson 1951). Furthermore, postmortem studies comparing Japanese with North Americans (Gore et al. 1964) and Ugandan natives with North Americans (Thomas et al. 1960) showed that the Japanese and Ugandan natives had a very low frequency of myocardial infarction and little evidence of thrombosis compared with North Americans. The great weakness of the disordered lipid-metabolism hypothesis, as Pickering (1964) has pointed out, is that it does not account for the thrombosis.

We have been trying to find a link between the two hypotheses. Platelet-aggregation, which has been proposed as the initial process in thrombus formation (Bizzozero 1882), can only be studied in aqueous media such as plasma. This limitation is a major technical obstacle when the effects of water-insoluble substances such as lipids are being studied. Furthermore many of the saturated fatty acids have high melting points and are difficult to maintain in solution at physiological temperatures. Plasma-lipids are transported as water-soluble lipoproteins and these lipoproteins contain many different classes of lipids in combination. The addition of lipoproteins as such does not permit an assessment of the effect of individual lipid classes on platelet-aggregation. We have tried to overcome these difficulties by incubating the individual lipids with plasma, red-blood cells, and albumin; but we have found that the most effective way of achieving physiological concentrations of lipids in a stable water-soluble form was to use the solubilising properties of phospholipids and prepare lecithin-sols of the fatty acids. We present our observations on the effect of these fatty-acid-lecithin sols, and also of phospholipids themselves, on platelet-aggregation.

Materials and Methods

Preparation of Phospholipids

Crude lecithin solution.—Crude lecithin 30 mg. was dissolved in a small volume of ether and 10 ml. of water was added slowly using a 'Vortex' mixer. The ether was evaporated by a stream of nitrogen. After filtration the final concentration of crude lecithin was 3 mg. per ml.; 0·5 ml. of this solution was added to 1·5 ml. platelet-rich plasma (P.R.P.).

Purified lecithin and other phospholipids.—Purified phospholipids were obtained from crude lecithin and from rat-liver phospholipids by silicic-acid chromatography. To obtain liver phospholipids, rat-liver was finely minced and the lipids extracted with 20 volumes of chloroform-ethanol (2:1 v/v) per gramme of tissue, the chloroform-methanol extract being washed with normal saline solution (Folch et al. 1957). Silicic acid was prepared by the method of Horning et al. (1960). After addition of lipid the column was eluted with 200 ml. chloroform in order to remove neutral lipids and elution was continued with increasing concentrations of methanol in chloroform (fig. 1); the eluant was collected in 7 ml. aliquots in an automatic fraction-collector. Monitoring of the eluant was done by determination of phosphorus in every third tube, using a modification of the Fiske-Subbarrow method by Barlett (1959). Under the conditions described the first phospholipid fraction is phosphatidic acid, the second, phosphatidylserine, and the third phosphatidylethanolamine. This is followed by lecithin in fraction 4 and sphingomyelin and lysolecithin in fractions 5 to 7. The purity of each fraction was checked by thin-layer chromatography on silica-gel G using conventional differential staining techniques. Good separation of phospholipids was obtained with the exception that sphingomyelin and lysolecithin were not completely separated in fractions 5 to 7.

Methanol and chloroform were evaporated from each phospholipid fraction and the residue was dissolved in 5 ml. ethanol

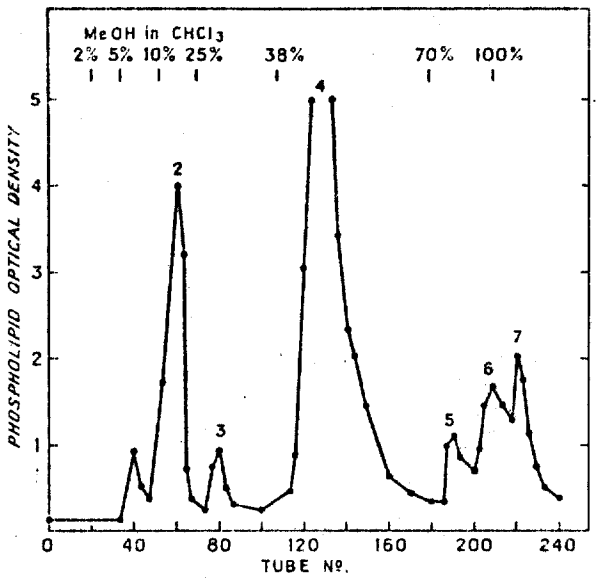

Fig. 1—7 phospholipid fractions obtained by silicic-acid chromatography of crude lecithin.
1.—Phosphatidic acid.
2.—Phosphatidyl serine.
3.—Phosphatidyl ethanolamine.
4.—Pure lecithin.
5–7.—Sphingomyelin and lysolecithin.

and stored at −20°C until required. When required the ethanol was evaporated from 1 ml. of the stock solution and the residue was dissolved in ether; 10 ml. of saline solution was added to the phospholipid solution in a vortex mixer. After filtration the final concentration of purified phospholipid was adjusted to 3 mg. per ml. For titration 0·5 ml. of each phospholipid fraction was added to 1·5 ml. P.R.P.

Preparation of Solutions of the Free Fatty Acids

The free fatty acids (F.F.A.) were dissolved in *N*/10NaOH and the pH was corrected to 7·8 (Shore and Alpers 1963). Solutions of F.F.A. were also prepared in 5% albumin in buffered saline solution.

In order to prepare fatty acid lecithin sols, about 10 mg. of the sodium salt of each fatty acid was powdered and slowly added to 10 ml. of purified lecithin solution using a vortex mixer. The mixture was shaken in a 'Vibro' mixer for 6 hours and the insoluble material removed by filtration. This gave a 0·0035*M* solution of each fatty acid and 0·5 ml. of each solution was added to 1·5 ml. P.R.P.

Other Materials

Blood—Blood was obtained from patients by venepuncture through stainless-steel needles into disposable plastic syringes and transferred to 'Polythene' test-tubes containing one-ninth the blood-volume of 3·8% (w/v) trisodium citrate.

Platelet-rich citrated plasma.—P.R.P. was obtained by centrifuging the polythene tube at 1500 r.p.m. for 10 minutes. The plasma was then pipetted into clean polythene test-tubes.

Platelet-free plasma (P.F.P.).—This was obtained in the same way as P.R.P. except that the rate of centrifugation was 3000 r.p.m. for 30 minutes.

Platelet-aggregation was carried out by the method of Born (1962) as modified by O'Brien (1962). 2 ml. human P.R.P. was added to a plastic cuvette containing a plastic coated magnetic stirrer. Using a red filter (608) in the titrator the light-intensity was adjusted till the optical-density scale was 0·40 on the galvanometer. Platelet-aggregation could be detected by a fall in optical density. When disaggregation occurred a rise in optical density followed. Stirring at low speed (100 r.p.m.) did not alter the optical density.

0·5 ml. of the lipid under test was added to 1·5 ml. of P.R.P. and the optical density was read at 1 minute intervals for 10 minutes. P.F.P. was used as a control to determine the effect of additions of the lipids on the optical density. After 10 minutes 1 or 2 μg. adenosine diphosphate (A.D.P.) in barbiturate-buffered saline solution (0·01 ml.) was added to the P.R.P. at room-temperature to assess the viability of the platelets.

The reproducibility of the method was tested by repeatedly adding 2 μg. A.D.P. to several samples of the one batch of P.R.P. and this gave optical densities with differences of less than 5%.

Platelet-aggregation with Mixtures of Phospholipids and F.F.A.

Linolenate-lecithin sol 0·5 ml. was added to 1·5 ml. P.R.P. in the titrator and after 10 minutes 0·5 ml. stearate-lecithin sol was added. Pure lecithin 0·5 ml. was added to 1·5 ml. P.R.P. in the titrator and after 10 minutes 0·5 ml. of stearate-lecithin sol was added. As a control 0·5 ml. stearate-lecithin sol was added to 1·5 ml. P.R.P., the volume having been increased to 2 ml. with P.F.P. A few microgrammes of sodium linolenate or sodium linoleate were added to palmitate-lecithin sol before addition to P.R.P.

Results

Phospholipid

When 0·5 ml. whole phospholipid sol (i.e., crude lecithin) was added to 1·5 ml. P.F.P. no fall in optical density was observed. When added to P.R.P. there was a marked fall in optical density and visible platelet-aggregation within 1 minute, reaching its peak in 3 minutes. Thereafter there was disaggregation, causing a rise in optical density. After 10 minutes the addition of 1 μg. A.D.P. led to further aggregation (fig. 2).

Phospholipid Fractions

Following fractionation by silicic-acid column-chromatography only fraction 4, lecithin, failed to cause platelet-aggregation (fig. 3). Fractions 1, 2, and 3 representing phosphatidic acid, phosphatidyl serine, and phosphatidyl ethanolamine respectively led to moderate platelet-aggregation; with fraction 5 to 7 (sphingomyelin plus lysolecithin) notable platelet-aggregation occurred. Furthermore the aggregation produced by sphingomyelin and lysolecithin appeared to be irreversible because the addition of A.D.P. 10 minutes later failed to induce any further change in optical density (fig. 3).

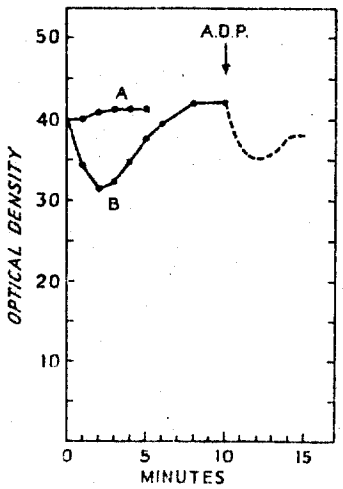

Fig. 2—Effect of crude lecithin on optical density of platelet-rich plasma (B) and platelet-free plasma (A). Means of 7 patients.

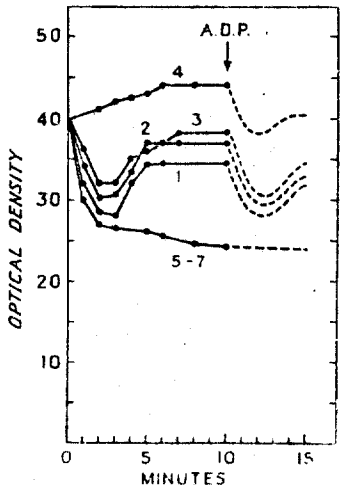

Fig. 3—Effect of purified phospholipids on platelet-rich plasma. For key, see fig. 1 (above). Mean of 4 patients.

Effect of F.F.A.

Of the several methods attempted to achieve a stable aqueous solution of F.F.A. only the sol with lecithin was satisfactory in that F.F.A. remained in solution at room-temperature and after centrifugation. With other methods the F.F.A. remained in solution at temperatures around 50°C but when added to P.R.P. it was impossible to determine how much the change in optical density was due to platelet-aggregation and how much to gel-formation.

Before making the fatty acid-lecithin sols each batch of purified lecithin was tested on P.R.P. since contamination with the other phospholipids, even in trace amounts, caused aggregation of platelets. The addition of these fatty-acid-lecithin sols to plasma did not change the pH of the plasma.

Saturated Fatty Acids

The effect of sols of lecithin with stearate, palmitate, and myristate on platelet-aggregation is shown in fig. 4a. Each sol caused platelet-aggregation but the degree of aggregation appeared to be related to the number of C-atoms in the chain. The fall in optical density was greatest with stearate and this change appeared to be irreversible since the subsequent addition of A.D.P. gave no further change in optical density.

Unsaturated Fatty Acids

Addition of the oleate-lecithin sol caused immediate but transient aggregation comparable with palmitate-lecithin and myristate-lecithin sols. Sols of lecithin with linoleate and linolenate produced no platelet-aggregation (fig. 4b).

Platelet-aggregation with Mixtures of Lecithin and Saturated and Unsaturated F.F.A.

Linolenate-lecithin sol added to P.R.P. before the addition of stearate-sol or palmitate-sol inhibited platelet-aggregation (fig. 5a). The same inhibitory effect was shown with linoleate-lecithin sol. The addition of lecithin also inhibited the aggregation caused by sols of lecithin with stearate and palmitate (fig. 5a). The addition of a few microgrammes of sodium linolenate to palmitate-lecithin sol before addition to P.R.P. partially inhibited the platelet-aggregation (fig. 5b); on the other hand the addition of sodium linoleate to palmitate-sol showed no inhibitory effect at all (fig. 5b). Neither sodium linolenate or sodium linoleate added direct to P.R.P. caused platelet-aggregation.

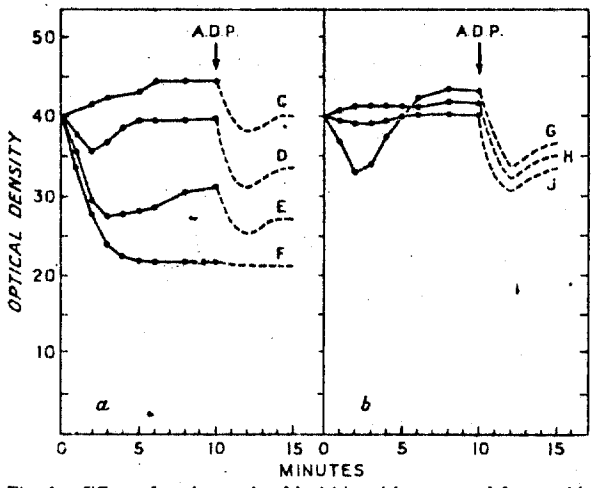

Fig. 4a—Effect of various sols of lecithin with saturated fatty acids on platelet-rich plasma. Means of 8 patients.
C.—No fatty acid.
D.—Myristate (14-carbon).
E.—Plamitate (16-carbon).
F.—Stearate (18-carbon).

Fig. 4b—Effect of various sols of lecithin with unsaturated fatty acids on platelet-rich plasma. Means of 8 patients.
G.—Oleate (18:1-carbon).
H.—Linolenate (18: 3-carbon).
J.—Linoleate (18: 2-carbon).

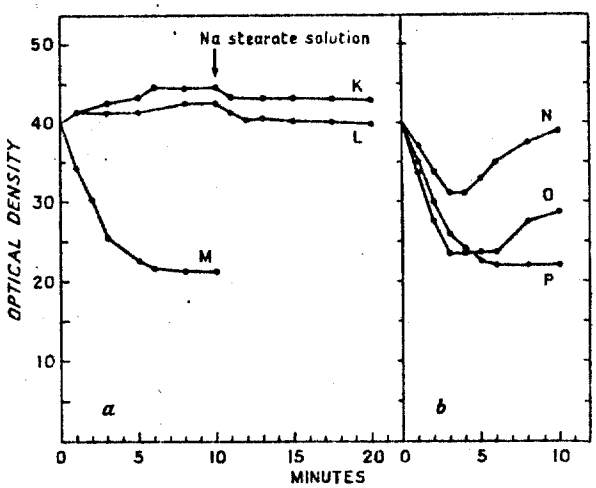

Fig. 5a—Inhibitory effect of sols on aggregation of platelet-rich plasma by stearate-sol. Means of 4 patients.
K.—Lecithin-sol followed by stearate-sol.
L.—Linolenate-lecithin sol followed by stearate-sol.
M.—Stearate-sol (control).

Fig. 5b—Effect of addition of sodium linolenate and sodium linoleate to palmitate-sol before mixing with platelet-rich plasma. Means of 4 patients.
N.—Sodium linolenate.
O.—Palmitate-sol alone (control).
P.—Sodium linoleate.

Discussion

There are now several studies using different approaches that show a link between lipids and platelet-aggregation. Mustard et al. (1962) attached flow-chambers to pigs and measured the deposits that formed following different diets. The greatest deposition was seen when the pigs were on high fat diets, particularly when the main source of dietary fat was egg-yolk. Electron microscopy proved these deposits to be masses of aggregated platelets. Conner and Poole (1961) showed that long-chain saturated fatty acids accelerated clotting in a Chandler (1958) rotating-tube. Conner et al. (1963) and Soloff and Wiedeman (1963) produced thrombosis in the vessels of the bat's wing and in dogs by injecting long-chain saturated fatty acids intravenously. In addition, this technique induced temporary thrombocytopenia in rabbits (Zbinden 1964). Using a turbidimetric technique similar to ours, Haslam (1964) found that behenate and other saturated fatty acids aggregated washed platelets. This worker also encountered difficulty in the stability of suspensions at 37°C. Shore and Alpers (1963) emphasised differences in effect between long-chain saturated fatty acids and unsaturated acids by estimating histamine-release from platelets. The saturated fatty acids induced platelet-damage whereas the polyunsaturated acids and short-chain saturated fatty acids caused little or no release of histamine. Owren et al. (1964) found that the adhesiveness of platelets to glass was reduced by linolenic acid but not by linoleic acid.

The F.F.A. common in human plasma and tissues have chains of 14 to 18 C-atoms. The melting points of the saturated acids and their poor solubility in aqueous solutions are the main difficulties in designing experiments to study their effect on platelets. These difficulties were overcome by using lecithin-sols so that we were able to test the range of fatty acids commonly found in human tissue fluids. Nevertheless, we cannot claim that our observations represent the in-vivo state as F.F.A. are more often bound to albumin and it seems obvious that in-vivo studies are necessary to confirm our observations. The concentration of F.F.A. used, however, was not beyond the range observed after certain stimuli in man. The final concentration was about 1000 μEq. per litre which is within the range reported in the fasting state in uncontrolled diabetes mellitus (Hales and Randle 1963, Schrade et al. 1963) and after catecholamine infusion (Duncan et al. 1965). Elevated F.F.A. have also been reported in animals under conditions of stress (Mallov and Witt 1960, Stoner 1962).

Our observations together with those described above suggest that a possible link between thrombosis and the lipid metabolic disorder in ischæmic heart-disease lies in the type of fatty acid with which the platelet comes into contact. The type of F.F.A. predominating in the plasma appears to be influenced by the nature of the dietary fat intake. Fleischman et al. (1964) showed that following a diet where the ratio of saturated to polyunsaturated fatty acids was 3:1, palmitic acid accounted for more than 50% of the serum F.F.A. On a diet where the ratio of saturated to polyunsaturated fatty acids was 1:3, the palmitic acid was only 18% of the diet. Our observations support the view of Owren et al. (1964) that linolenic acid may protect platelets since the addition of this substance to plasma inhibited the platelet-aggregation expected following the addition of saturated acids.

We do not know how far the effect of different phospholipids is dependent on the nature of its constituent fatty acid. Lecithin by itself produced no platelet-aggregation and under certain circumstances appeared to protect platelets from the subsequent effects of saturated fatty acids. Lecithin forms 75% of the plasma-phospholipids. More than 50% of the fatty acids esterified with lecithin are unsaturated acids. Our fractions 5 to 7 (sphingomyelin and lysolecithin) produced irreversible aggregation. Sphingomyelin is esterified almost entirely with saturated fatty acids and is the most abundant phospholipid in the atheromatous plaque (Bottcher 1964, Smith 1965). Although phospholipids have been studied in blood-coagulation systems (Poole and Robinson 1956, Billimoria et al. 1965) we are unaware of any other studies on phospholipids and platelet-aggregation with which our results can be compared. In our system the increase in phospholipid concentration of the plasma is of the order of 75 mg. per 100 ml. This is not unduly high for lecithin but would be beyond the usual range for the other phospholipids. It is notable, however, that contamination of our lecithin fraction with only trace amounts of other phospholipids led to immediate platelet-aggregation.

Summary

Sodium salts of free fatty acids (F.F.A.) were dissolved in lecithin purified by silicic-acid chromatography to form a 0·0035 *M* solution. Platelet-aggregation was observed by adding 0·5 ml. of each phospholipid or F.F.A. solution to platelet-rich plasma. Pure lecithin failed to cause platelet-aggregation. Phosphatidic acid, phosphatidyl serine, and phosphatidyl ethanolamine caused reversible aggregation but aggregation caused by sphingomyelin and lysolecithin was irreversible. Of the F.F.A.'s tested, only linoleate and linolenate did not cause platelet-aggregation. Both lecithin and linolenate inhibited the platelet-aggregation induced by saturated fatty acids.

We acknowledge a gift of linolenic acid from Dr. W. F. T. Cuthbertson and a gift of linoleic acid from Dr. J. Blain.

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✓

Pathology

CYTOTOXIC EFFECT OF SODIUM OLEATE. R. Kinosita, H. Miyaji,* and T. Kakefuda.* City of Hope Medical Center, Duarte, Calif.

One intraperitoneal injection of 10 mg sodium oleate in 1 ml water caused hitherto undescribed changes in the liver of the rat. Within a few days, the liver became plump with rounded edges. Cytoplasm of the liver cells contained unstained patches of irregular shape. Electron microscopy of these areas demonstrated the lack of ribosomes and alteration of the endoplasmic reticulum, which, instead of cristae, formed a network of narrow canals or became fuzzy and filamentous. The cristae and membranes of mitochondria were indistinct. Eventually, the liver cells regained their usual structures. The liver remained plump in form, unaccompanied by cirrhotic changes. Marked dilatation of the central veins and hyperplasia of the liver cells was evident. Females were more sensitive to the compound than males. Fatty acids with 8 or more carbons were effective; those with 12 and 14 carbons were most active. Saturation and hydroxylation altered the results very little.

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MECHANISM OF THE CLOT-PROMOTING EFFECT OF FATTY ACIDS AND SYNTHETIC ANIONIC DETERGENTS

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(Received for publication March 17, 1961)

In 1916, Stuber and Heim observed that certain fatty acids shorten the recalcification time of oxalated horse plasma. During recent years, some attention has again been paid to this problem because it was thought that the increased coagulability, which is observed after a fatty meal, might be caused by an increased concentration of free fatty acids in the blood.

Recent studies on the subject have been published by Poole (1955). She observed that sodium palmitate, sodium stearate, sodium oleate, and sodium laurate were able to shorten the clotting times of human plasma both with and without the presence of Russell's viper venom (R.V.V.). O'Brien 1957 could demonstrate this activity of the fatty acids only in the presence of R.V.V. Pilkington (1957), who worked with recalcification tests without the presence of R.V.V., found the unsaturated fatty acids oleic, linoleic, linolenic acids, and the saturated acids with 12 and 14 carbon atoms to be inactive. On the other hand, he found that the saturated acids with 16–20 carbon atoms were active.

The main purpose of the present work has been:

1. to investigate the importance of the length of the hydrocarbon chain of the saturated fatty acids for their clot-promoting activity,

2. to determine whether unsaturated fatty acids and some other anionic substances with a hydrophobic tail promote the clotting,

3. to study some details concerning the mechanism by which these substances accelerate the thrombin formation.

As it has been shown in previous work (Korsan-Bengtsen, 1960) that fatty acids with a long hydrocarbon chain inactivate thrombin and prolong the thrombin-fibrinogen clotting time, the present study has been concentrated on their effect on the thrombin generation. For this study it was necessary to use special clotting systems. The composition of these systems and the preparation of their components will be presented.

MATERIALS AND METHODS

Accelerin: (Serum Ac-globulin) was prepared by a modification of the method of Cox, Lanchantin & Ware (1955). The details of this method are described elsewhere in this journal (Korsan-Bengtsen & Ygge, 1961).

Adsorbed plasma: Human oxalated plasma, collected as described below, was adsorbed with $BaSO_4$ C.P. 100 mg/ml.

Buffered physiological saline: 4 volumes of physiological saline + 1 volume of the isotonic veronal buffer of Owren, pH = 7.3, (Owren 1947).

Cholate: Sodium cholate puriss from E. Merck AG., Darmstadt, Germany.

Clotting technique: The tests were performed in small thin-walled test tubes, 9 x 35 mm in size, which were continuously tilted in a thermostat-regulated water bath at 37° C ± 1° C and observed for clotting. The time was measured with ordinary stop watches.

Collection of plasma: Blood was collected from the antecubital vein with coarse and sharp silicone-treated needles into lusteroid tubes and mixed with 1/10 its volume of 3 per cent trisodium citrate or 2 per cent dipotassium oxalate. Centrifugation was performed in an "International" refrigerated centrifuge (model PR-2) at 1500 R.C.F. at 4° C for 30 minutes.

Dinonylsulfosuccinate: The commercial compound "Manoxol 100 % pure" from Hardman Holden Ltd., Manchester 10, England.

Dodecylbenzenesulfonate: The same substance which was used in a previous paper (Korsan-Bengtsen 1960).

Eluate: 200 ml human oxalated plasma was adsorbed with $BaSO_4$ (100 mg/ml). After washing the $BaSO_4$ three times with buffered physiological saline, the elution was performed with 20 ml 6 per cent trisodium citrate. The eluate was dialyzed one hour against running tap-water and then for two days at + 4° C against 4000 ml of distilled water containing 0.05 per cent of trisodium citrate. The dialysis fluid was changed five times. After the dialysis, the eluate was freeze-dried. The dry substance was powdered and extracted three times with 100 ml portions of chloroform-methanol 3:1 at - 20° C. The powdered eluate was allowed to be in contact with the chloroform-methanol solution for 30 minutes every time. After the last extraction the powder was taken directly to a vacuum exsiccator and dried. It was kept in a stoppered tube at - -20° C.

Before use, it was dissolved in a volume of buffered physiological saline corresponding to 1/10 the volume of the eluate before it was freeze-dried. The small quantity of the dried eluate which was insoluble, was removed by centrifugation. This final solution is henceforth referred to as "the eluate". It contained approximately 0.5 per cent trisodium citrate, and had a prothrombin activity which was about 50 times that of normal human plasma, tested with the prothrombin determination method of Hjort, Rapaport, and Owren (1955). In most of the experiments, dilutions of this "stock eluate" were used. The dilutions were made with buffered physiological saline containing 0.5 per cent trisodium citrate.

Fatty acids: The same substances which were used in a previous paper (Korsan-Bengtsen 1960).

Fibrinogen: Human fibrinogen prepared by Blombäck & Blombäck's method (1956) delivered by Kabi AB, Stockholm, Sweden. Before use, it was dialyzed against buffered physiological saline for 48 hours.

Gangliosides: The same substance which was used in a previous paper (Korsan-Bengtsen 1960).

Lipid material: Human brain, washed and ground, was treated 6 times with an excess of acetone. 1 g of the dry powder was extracted with 60 ml chloroform C.P. The chloroform was evaporated, and the residue (330 mg) suspended in 50 ml of buffered physiological saline. It was kept in small test tubes at - -20° C.

Lysed platelets: 90 ml of blood was collected in a lusteroid tube and mixed with 10 ml of a 2 per cent solution of disodium sequestrene (disodium ethylenediaminetetraacetate Merck AG., Darmstadt, Germany). After centrifugation for 10 minutes at 170 R.C.F. in an "International" refrigerated centrifuge (model PR 2), the platelet-rich plasma was collected and recentrifuged for 5 minutes. The

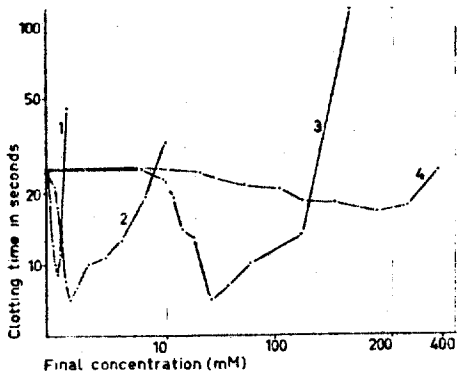

Fig. 1. Effect of sodium dodecylbenzenesulfonate, sodium laurate, sodium caprinate, and sodium caprylate on the clotting times of plasma in the presence of R.V.V.

Clotting system: 0.2 ml human citrated, platelet-poor plasma + 0.2 ml R.V.V. diluted 1/10000 with buffered physiological saline + 0.2 ml of the different fatty acids or sodium dodecylbenzenesulfonate of various concentrations. After 5 minutes incubation at 37° C, 0.2 ml $CaCl_2$ 0.025 M was blown down and the clotting time recorded.

Abscissa: final concentration of the different fatty acids and sodium dodecylbenzenesulfonate. Ordinate: logarithmic scale.

1 = sodium dodecylbenzenesulfonate.
2 = sodium laurate.
3 = sodium caprinate.
4 = sodium caprylate.

platelets were then sedimented at 1500 R.C.F. for 30 minutes, and washed 3 times in buffered physiological saline by resuspension and recentrifugation. Finally, they were suspended in 5 ml of buffered physiological saline and kept at −20° C until the day they were used. Before use the lysed platelets were heated to 60° C for 15 minutes, after which no proaccelerin activity was left in the solution.

Proaccelerin (fact. V) was prepared as described by Hjort (1957). The details of this method are described elsewhere in this journal (Korsan-Bengtsen & Ygge, 1961).

Proaccelerin determination method: A modification of the one-stage method of Aas (1952) was used. (Korsan-Bengtsen & Ygge, 1961).

Russell's viper venom (R.V.V.): Stypven, Burroughs Wellcome & Co., London, England.

Thrombin: Human thrombin prepared as described elsewhere in this journal (Korsan-Bengtsen & Ygge, 1961).

EXPERIMENTS AND RESULTS

Effect of Fatty Acids with a Varying Length of the Hydrocarbon Chain and of Dodecylbenzenesulfonate on the Clotting of Plasma in the Presence of R.V.V.

The experiments were performed by mixing 0.2 ml of human citrated, platelet-poor plasma with 0.2 ml R.V.V. (1/10000) and 0.2 ml of the different substances to be tested in various concentrations. After 5 minutes of incubation at 37° C, 0.2 ml $CaCl_2$ 0.025 M was blown down and the clotting times recorded.

The results are illustrated in Fig. 1. Rather low concentrations of sodium laurate and sodium dodecylbenzenesulfonate shorten the clotting times. Caprinate also shortens the clotting times to the same degree, but the concentration needed is about ten times that of laurate. Caprylate gives a slight decrease in the clotting times in very high concentrations.

It has also been shown in similar experiments that the sodium salts of the unsaturated fatty acids, oleic and linoleic acids, shorten the clotting times to the same degree in the same concentrations as laurate. The synthetic detergent dinonylsulfosuccinate behaves as dodecylbenzenesulfonate. Furthermore, sodium cholate and gangliosides have been tested and were also found to shorten the clotting times.

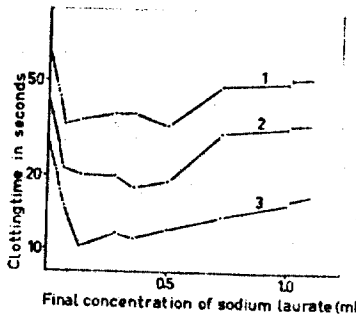

Fig. 2. Clot-promoting effect of sodium laurate in clotting mixtures with a high concentration of proaccelerin and different concentrations of eluate.

Clotting system: 0.1 ml eluate of different concentrations + 0.1 ml proaccelerin + 0.1 ml fibrinogen 1% + 0.1 ml R.V.V. 1/10000 + 0.1 ml physiological saline + 0.1 ml sodium laurate of different concentrations or physiological saline as a control + 0.1 ml $CaCl_2$ 0.04 M.

Ordinate: logarithmic scale.

1: eluate diluted 1:12 with buffered physiological saline.

2: eluate diluted 1:5 with buffered physiological saline.

3: eluate undiluted.

Inability of Sodium Laurate and Sodium Dodecylbenzenesulfonate to Promote the Clotting in Clotting Systems Containing Lysed Platelets or Lipid Material from Brain.

According to Rapaport, Aas & Owren (1954), R.V.V. together with a lipid material has an activity in the blood coagulation which is equivalent to convertin (SPCA). When R.V.V. in a sufficient concentration is added to plasma, the thrombin generation, thus depends upon the content in the plasma of prothrombin (fact. II), proaccelerin (fact. V), Stuart factor (fact. X), lipid material, calcium, and inhibitors. Consequently, an increase in the thrombin generation can be caused by an increased activity of one or several of these clot-promoting substances or by a decreased activity of inhibitors.

In the following experiments performed with clotting systems containing R.V.V. it will be shown that sodium laurate has no accelerating effect on the clotting when lysed platelets or lipid material from brain is present. This is the case both with low and high concentrations of eluate and proaccelerin in the clotting systems. However, without lysed platelets or lipid material present, an accelerating effect of sodium laurate is demonstrable at all tested concentrations of eluate and proaccelerin.

The first experiment is shown in Fig. 2. There was a high concentration of proaccelerin in the clotting system, and a varying concentration of the eluate obtained from human plasma as described in the section "Materials and methods". As will be seen, a high concentration of eluate did not eliminate the effect of the fatty acid. Quite the contrary, the fatty acid was more active when the concentration of eluate was high.

Fig. 3 shows an experiment with the same clotting mixture but an optimal concentration of lysed platelets had been added. There was no shortening of the clotting times with sodium laurate at any concentrations. The same results was obtained in a similar experiment when using lipid material from brain instead of lysed platelets. Sodium dodecylbenzenesulfonate had the same effects as sodium laurate.

Experiments have also been performed with an optimal concentration of eluate and varying concentrations of proaccelerin in the clotting mixtures. Sodium laurate and sodium dodecylbenzenesulfonate increased the coagulability at all concentrations of proacce-

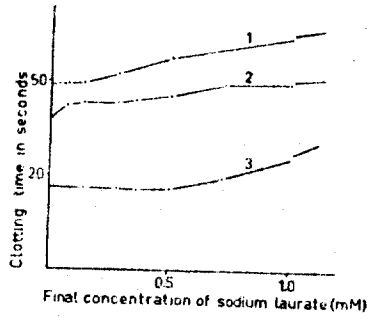

Fig. 3. Clot-promoting effect of sodium laurate in clotting mixtures with a high concentration of proaccelerin and a high concentration of lysed platelets and with different concentrations of eluate.

Clotting system: 0.1 ml eluate of different concentrations + 0.1 ml proaccelerin + 0.1 ml fibrinogen 1% + 0.1 ml R.V.V. 1/10000 + 0.1 ml lysed platelets + 0.1 ml sodium laurate of different concentrations or physiological saline as a control + 0.1 ml $CaCl_2$ 0.04 M.

Ordinate: logarithmic scale.

1: eluate diluted 1:100 with buffered physiological saline.

2: eluate diluted 1:40 with buffered physiological saline.

3: eluate diluted 1:12 with buffered physiological saline.

lerin. However, when lysed platelets or lipid material from brain was present there was no shortening of the clotting times with sodium laurate or sodium dodecylbenzenesulfonate.

The conclusion which might be drawn from these experiments is that sodium laurate and sodium dodecylbenzenesulfonate either have an effect on the coagulation similar to lysed platelets and lipid material from brain or that lysed platelets and lipid material from brain inhibit the effect of sodium laurate and sodium dodecylbenzenesulfonate.

Effect of Dodecylbenzenesulfonate in a Lipid-Poor Thrombin Generation System.

In the next experiment, it will be shown that the effect of dodecylbenzenesulfonate on the clotting is not identical to the effect of the lipid material or lysed platelets. It was necessary to produce a lipid-poor thrombin generation mixture for this demonstration.

As probably very small quantities of lipid material are needed for the thrombin formation, it is impossible to obtain an ideal lipid-free system. Our aim has been to obtain clotting systems which form no or very little thrombin without addition of extra lipid. On the other hand, there has to be a rapid thrombin formation in the system when a sufficient concentration of lipid material is added.

The clotting system used in the experiment shown in Fig. 4 was a two-stage system. The mixture in which thrombin was expected to be formed was composed as follows:

1. Eluate which had been extracted with chloroform-methanol as described above.
2. R.V.V.
3. Accelerin or proaccelerin prepared as described above.
4. Buffered physiological saline, lipid material (1.2 mg/ml), or dodecylbenzenesulfonate of various concentrations.
5. $CaCl_2$ 0.03 M.

From this incubation mixture, aliquots of 0.2 ml were taken and blown down into 0.2 ml fibrinogen 0.25 per cent at the times indicated in the figure.

The results of this experiment can be summarized as follows:

1. There was a minimal formation of thrombin in the system with accelerin with-

out extra lipid added. When lipid was added to this system, thrombin formed sufficiently and rapidly. Dodecylbenzenesulfonate was, on the other hand, not able to influence the thrombin formation.

2. If proaccelerin was used in the system instead of accelerin, the thrombin formation still was very poor. However, in this system dodecylbenzenesulfonate in a sufficient concentration was able to accelerate the thrombin formation almost to the same degree as the lipid material.

Numerous such experiments have been performed with many different concentrations of sodium laurate or dodecylbenzenesulfonate and also with lysed platelets instead of the lipid material. The result illustrated in Fig .4 could always be equalled.

The main conclusion which may be drawn from this experiment is that the effect of dodecylbenzenesulfonate is not identical to the effect of the lipid material, because dodecylbenzenesulfonate did not increase the thrombin formation in the accelerin system whereas lipid material had a great accelerating effect.

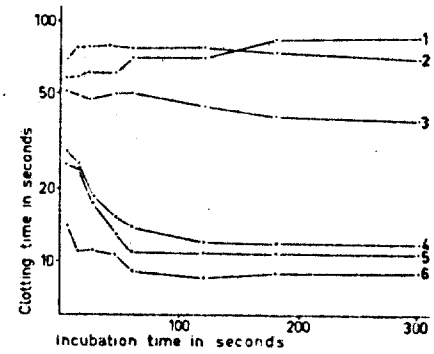

Fig. 4. Effect of dodecylbenzenesulfonate in a lipid-poor thrombin generation system with purified accelerin or proaccelerin present.

Clotting system: 0.5 ml eluate diluted 1:15 + 0.5 ml lipid material from brain. 1.2 mg per ml, dodecylbenzenesulfonate 0.14 mM, or physiological saline respectively + 0.5 ml proaccelerin or accelerin + 0.5 ml R.V.V. 1/10000 dissolved in 0.03 M $CaCl_2$.

From these incubation mixtures 0.2 ml was removed after the times indicated and blown down into 0.2 ml fibrinogen 0.25 %.

Ordinate: logarithmic scale.

1: Purified accelerin and physiological saline in the incubation mixture.
2: Purified accelerin and dodecylbenzenesulfonate in the incubation mixture.
3: Proaccelerin and physiological saline in the incubation mixture.
4: Proaccelerin and dodecylbenzenesulfonate in the incubation mixture.
5: Purified accelerin and lipid material in the incubation mixture.
6: Proaccelerin and lipid material in the incubation mixture.

Effect of Dodecylbenzenesulfonate in a Thrombin Generation System with "Crude Accelerin".

The most plausible explanation of the result of the experiment shown in Fig. 4 at first seemed to be that dodecylbenzenesulfonate activates proaccelerin to accelerin. Some observations which were made during the preliminary work spoke against this theory. First, we were never able to demonstrate any increased activity in plasma or proaccelerin preparations with the specific proaccelerin determination method after addition of dodecylbenzenesulfonate or laurate. Secondly, we were unable to increase the thrombin formation in a lipid-poor system very much by increasing the accelerin activity a hundred times. Further, the thrombin

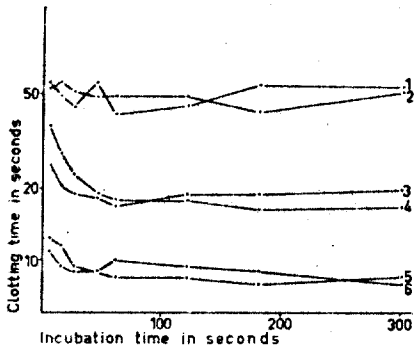

Fig. 5. Effect of dodecylbenzenesulfonate in a thrombin generation system with "crude accelerin". Clotting system: 0.5 ml eluate diluted 1:15 + 0.5 ml lipid material from brain, 1.2 mg per ml, dodecylbenzenesulfonate 0.14 mM, or physiological saline respectively + 0.5 ml proaccelerin or "crude accelerin" + 0.5 ml R.V.V. 1:10000 dissolved in 0.03 M $CaCl_2$.

From these incubation mixtures 0.2 ml was removed after the times indicated and blown down into 0.2 ml fibrinogen 0.25 %.

Ordinate: logarithmic scale.

1. "Crude accelerin" + physiological saline in the incubation mixture.
2. Proaccelerin + physiological saline in the incubation mixture.
3. "Crude accelerin" + dodecylbenzenesulfonate in the incubation mixture.
4. Proaccelerin + dodecylbenzenesulfonate in the incubation mixture.
5. "Crude accelerin" + lipid material in the incubation mixture.
6. Proaccelerin + lipid material in the incubation mixture.

generation mixture used contained R.V.V. which has been shown by Hjort (1957) to active proaccelerin into accelerin. The following experiment also definitely speaks against the theory.

A thrombin concentration which gave maximal activation of the proaccelerin preparation used was tested. It was found that 1 N.I.H. thrombin unit per ml was sufficient to activate the dilution of the proaccelerin-preparation used. No further increase in activity could be obtained by the addition of more thrombin. An experiment was now performed exactly as described in Fig. 4 but with this "crude accelerin" instead of the purified accelerin preparation. Fig. 5 shows the result. The accelerating effect of dodecylbenzenesulfonate was, as will be seen, quite as good with crude accelerin as with proaccelerin.

The conclusion is that there is something present in the proaccelerin and "crude accelerin" preparations which is necessary for the accelerating effect of dodecylbenzenesulfonate. This component is removed by the purification of accelerin.

Experiment with a Thrombin Generation Mixture without R.V.V. Present.

Fig. 6 shows an experiment with a two-stage system of exactly the same composition as that shown in Fig. 4 but without R.V.V. present. The experiment shows that. R.V.V. is not necessary for the accelerating effect of dodecylbenzenesulfonate on the thrombin formation. Similar experiments have been performed with sodium laurate with the same result.

DISCUSSION

It has been known for many years that in the cephalin fraction prepared from brain and other organs there are clot promoting substances very similar to those present in platelets. During recent years (Wolf, 1956, O'Brien, 1956) it has also been shown that

purified and synthetic ethanolaminglycerophospholipid and seringlycerophospholipid are active as clot-promoting agents (Poole & Robinsson, 1956, Biggs & Bidwell, 1957). The great attention which has been paid to this subject is probably due to the fact that this is one of the few clotting factors which is relatively well chemically characterized.

The phospholipids which have been shown to have activity in the blood coagulation are acid phospholipids. Our working hypothesis at the beginning of this work was that the fatty acids might substitute the glycerophospholipid, which in turn would mean that the phospholipid effect is rather unspecific. The experiments show that the effect of the fatty acids is not identical to the effect of the lipid material from brain or lysed platelets.

Contrary to the lipid material, the fatty acids are inactive in a system where purified accelerin instead of a "crude accelerin" preparation is used. The reasonable explanation is that in the "crude accelerin" there is a substance present which is necessary for the accelerating effect of the fatty acids.

Of the known clotting factors which are of importance for the thrombin formation in a system containing R.V.V., the proaccelerin and crude accelerin preparations contain proaccelerin-accelerin, small amounts of lipids, and perhaps inhibitors. Therefore it seems logical to discuss the following possibilities to explain the clot-promoting activity of sodium laurate and dodecylbenzenesulfonate.

1. Proaccelerin is activated by the anions. This is not probable for the following reasons. First no increased proaccelerin activity could be registered by the proaccelerin determination method after addition of sodium laurate or dodecylbenzenesulfonate to the proaccelerin preparation. Second, the clot accelerating effect of the anions was quite as good after as before the activation of pro-

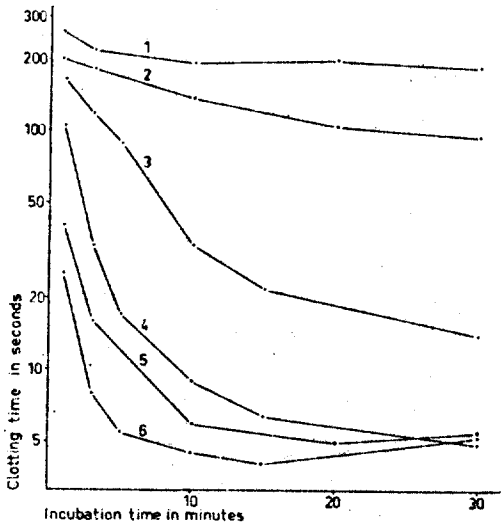

Fig. 6. Effect of dodecylbenzenesulfonate in a thrombin generation system without R.V.V.

Clotting system: 0.5 eluate undiluted + 0.5 ml proaccelerin or accelerin + 0.5 ml lipid material, 1.2 mg per ml, dodecylbenzenesulfonate 0.28 mM or buffered physiological saline respectively + 0.5 ml $CaCl_2$ 0.03 M.

After the times indicated, 0.2 ml of these incubation mixtures was removed and blown down into 0.2 ml fibrinogen 0.25 %.

Ordinate: logarithmic scale.

1: Accelerin + physiological saline in the incubation mixture.

2: Accelerin + dodecylbenzenesulfonate in the incubation mixture.

3: Proaccelerin + physiological saline in the incubation mixture.

4: Proaccelerin + dodecylbenzenesulfonate in the incubation mixture.

5: Accelerin + lipid material in the incubation mixture.

6: Proaccelerin + lipid material in the incubation mixture.

accelerin with thrombin. Third, the clotting times in the system with purified accelerin were never shortened appreciably ewen when the accelerin activity was increased a hundred times.

2. The small amounts of lipid material present in the preparations is activated by the anions, for instance by splitting of lipoprotein complexes.

3. The fatty acid and dodecylbenzenesulfonate combine with a co-factor present in the proaccelerin preparation to give an activity similar to that of the lipid material.

4. The anions neutralize a lipid factor inhibitor.

SUMMARY

In a one-stage clotting mixture with human citrated, platelet-poor plasma and Russell's viper venom, the sodium salts of lauric acid, capric acid, caprylic acid, oleic acid, linoleic acid, cholic acid, gangliosides, and the synthetic detergents sodium dodecylbenzenesulfonate and sodium dinonylsulfosuccinate are able to promote the clotting.

Sodium laurate and sodium dodecylbenzenesulfonate are not able to influence the thrombin formation in lipid-poor thrombin generation systems when purified accelerin prepared by anion exchange resin chromatography is used. On the other hand if proaccelerin or "crude accelerin" instead of purified accelerin is used in these systems, sodium laurate and sodium dodecylbenzenesulfonate, accelerate the thrombin formation considerably. Lipid material from brain and lysed platelets have a high and similar activity in both systems.

The conclusion which has been drawn is that the effect of the fatty acids and synthetic detergents on the thrombin generation is not identical to the effect of lysed heated platelets or lipid material from brain. However, together with a substance present in the proaccelerin and "crude accelerin" preparations used, the fatty acids and synthetic detergents have an activity similar to lipid material and lysed heated platelets.

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EFFECT OF PROLONGED INTRAVENOUS INFUSION OF OLEATE SODIUM ON SPONTANEOUS AND HISTAMINE STIMULATED GASTRIC SECRETION IN RATS

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The inhibitory effect of dietary fat on gastric secretion has been described over one hundred years ago (EWALD and BOAS, 1866). According to present opinion, the inhibition of both secretion and motility of the stomach occurs in the presence of fat in the proximal small intestine, particularly in the duodenum. This effect of fat is considered mediated by a humoral agent present in the mucosa of the upper intestinal tract (WOODWARD, 1957; MENGUY, 1959; LONG and BROOKS, 1960; KONTUREK and GROSSMAN, 1965; BIBLER *et al.*, 1966; SWAN *et al.*, 1966; GREGORY, 1967; LANDOR *et al.*, 1967). Most previous studies on inhibition of gastric function by fats, were concerned with an injection of natural oils into a segment of the upper gastrointestinal tract and with the immediate action of such an injection of oils on gastric secretion and/or motility. We found however, that feeding rats with a fat rich diet for several months induced significant inhibition of nonstimulated or histamine stimulated gastric secretion (KOWALEWSKI, 1969). In these studies the contact of fat with duodenal mucosa appeared to be a necessary condition for the production of gastric inhibition. This may not be true in light of more recent experiments demonstrating that intravenous emulsions of fat had inhibitory action on gastric secretion (BAUME *et al.*, 1964 and 1966). It was shown that natural oils have lesser inhibitory action on gastric secretion than their products of lypolysis, fatty acids and soaps. ROCHMAN *et al.* (1969) injected some fatty acids intravenously or intraperitoneally into rats with ligated pylorus and found a significant decrease of the volume of gastric juice collected four hours after the Shay operation. Best results were obtained with an injection of sodium

oleate (ROCHMAN *et al.*, 1969). These authors did not analyse qualitatively the collected gastric secretion. This interesting work (ROCHMAN *et al.*, 1969) stimulated our present study.

We did not consider the Shay operation (SHAY *et al.*, 1945) as a method of choice for the study of an inhibitory factor of gastric secretion. It is known that ligation of pylorus is followed by post-surgical hyperacidity, due to the stimulation of pressure receptors in the antral mucosa (BRODIE, 1966). We felt that a gastric fistula in rats offers a more convenient and precise tool for the study of gastric secretion (BEL *et al.*, 1966; KOWALEWSKI and CHMURA, 1969). We found previously that chronic gastric fistula combined with chronic intravenous cannula permits repeated and prolonged studies of gastric function during the intravenous administration of secretory stimulants or inhibitors (KOWALEWSKI, 1967*a* and *b*; KOWALEWSKI and CHMURA, 1968 and 1969).

In the experiment described in our present report, we used rats bearing gastric and intravenous cannulas. Sodium oleate was infused intravenously for 24 hours with or without 'maximal' dose of histamine, and gastric juice was collected during the infusion period.

METHODS

Male Wistar rats were used in this study. Surgery was performed under ether anaesthesia after 24 hours fasting. Chronic gastric fistula was produced in each rat and a miniature stainless steel cannula was implanted in the anterior wall of the rumen. At the same operation a polyethylene tube was implanted in the vena cava. These methods were described in detail recently (KOWALEWSKI and CHMURA, 1969). Secretion studies were performed in all rats three weeks after surgery. Prior to the collection of gastric juice, rats were fasted for 24 hours, but water was allowed as desired. On the morning of the experiment each rat was placed in a modified Bollman cage (BOLLMAN, 1948), and the stopper screw of the cannula was removed for the initial drainage of gastric content for 30 minutes. This first sample was discarded. Intravenous infusion and simultaneous collection of gastric juice was begun. The infusion rate, in all animals, was 120 ml/kg body weight for 24 h. Following solutions were used for infusion: (1) normal saline; (2) normal saline containing sodium oleate: 30 ml of 0.2 N sodium oleate mixed with 90 ml of normal saline; (3) histamine in normal saline: histamine dihydrochloride, 100 mg in 120 ml of saline; (4) both sodium oleate and histamine in normale saline in doses similar to those used in previous solutions. We found previously (KOWALEWSKI and CHMURA, 1968) that 100 mg of histamine dihydrochloride per kg body weight/24 hours, administered intravenously, is the 'maximal' dose, for the stimulation of parietal cells in rats bearing permanent gastric fistulas. Gastric juice was titrated in 1.0 or 0.5 ml aliquots, using a Radiometer type TTT1 Titration Assembly, and Type ABU 1 Auto Burette Unit. The pH was read and the sample was then titrated with 0.1 N NaOH to pH 7. Chlorides were determined by the Coulometric-Amperometric titration of silver ions using the Buchler-Cotlove Chloridometer. Sodium and potassium were analyzed with a flame photometer. Pepsin was determined by a modified Anson's method (AITKEN *et al.*, 1954). The biochemical results were evaluated statistically by the Students' *t* test.

RESULTS

Table I summarizes the effect of the infusion of sodium oleate and/or histamine on the output of various components of gastric juice. Review of this table and the analysis of the significance of the determinations permits us to conclude that sodium oleate resulted in the suppression of volume of gastric secretion and of the output of acid and pepsin in nonstimulated and in histamine stimulated rats. Regarding the statistical evaluation of the data the most important is the comparison of Group I with Group 2 and of Group 3 with Group 4.

By comparing Table I with Table II one can appreciate the importance of volume of gastric secretion on the output of the parameters studied. Table II summarizes the data on the concentration of gastric juice components in four groups of rats. It is apparent from the analysis of this table, that sodium oleate infusion did affect the concentration of both Na^+ and Cl^- in the gastric secretion. This is probably due to the presence of these electrolytes in the infused solution of sodium oleate. Histamine affected the concentration of HCl, as it was expected. Sodium oleate infusion did not significantly alter the [illegible] or pepsin.

TABLE I. *Effect of intravenous infusion of sodium oleate and/or histamine on 24 h output of various components of gastric juice in rats.* Av. values and S.D.

Group	Infusion	No. of rats	B.W. g	Vol. ml	HCl mEq	Na^+ mEq	K^+ mEq	Cl^- mEq	Pepsin mg
1	Saline	10	232 ±6	14.1 ±3.8	0.97 ±0.4	0.70 ±0.19	0.25 ±0.09	0.91 ±0.18	362 ±174
2	Sodium oleate in saline	10	231 ±5	7.0 ±2.0	0.38 ±0.2	0.51 ±0.20	0.12 ±0.05	0.81 ±0.33	232 ±120
3	Histamine in saline	10	232 ±16	23.9 ±7.3	2.69 ±1.0	0.76 ±0.28	0.25 ±0.08	1.59 ±0.68	578 ±230
4	Histamine and sodium oleate in saline	15	230 ±16	7.4 ±6.5	0.72 ±0.7	0.40 ±0.38	0.22 ±0.30	1.32 ±0.95	186 ±178
P	Group 1 *versus* 2		NS	<0.01	<0.01	<0.05	<0.01	NS	<0.05
	Group 1 *versus* 3		NS	<0.01	<0.01	NS	NS	<0.01	<0.05
	Group 1 *versus* 4		NS	<0.01	NS	<0.05	NS	NS	<0.05
	Group 2 *versus* 3		NS	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01
	Group 2 *versus* 4		NS	NS	NS	NS	NS	NS	NS
	Group 3 *versus* 4		NS	<0.01	<0.01	<0.05	NS	NS	<0.01

NS = not significant, $P > 0.05$.

TABLE II. *Effect of intravenous infusion of sodium oleate and/or histamine on the concentration of various components of gastric juice, collected or 24 h in rats.* Same rats as in Table I. Av. values and S.D.

Group	Infusion	pH	mEq/l HCl	mEq/l Na^+	mEq/l K^+	mEq/l Cl^-	Pepsin mg/ml
1	Saline	1.68 ±0.57	66 ±20	51.5 ±13.0	17.3 ±3.8	68.0 ±17.5	25.5 ±10.6
2	Sodium oleate in saline	3.18 ±2.10	52 ±31	75.9 ±25.4	17.0 ±5.7	114.0 ±20.2	32.7 ±12.0
3	Histamine in saline	1.21 ±0.05	112 ±20	33.8 ±15.5	10.8 ±2.6	66.6 ±22.7	23.3 ±3.8
4	Histamine and sodium oleate in saline	1.52 ±0.50	96 ±35	58.1 ±29.7	14.3 ±8.6	164.0 ±8.6	21.4 ±8.0
P	Group 1 *versus* 2	<0.05	NS	<0.01	NS	<0.01	NS
	Group 1 *versus* 3	<0.05	<0.01	<0.01	<0.01	NS	NS
	Group 1 *versus* 4	NS	<0.05	NS	NS	<0.01	NS
	Group 2 *versus* 3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05
	Group 2 *versus* 4	<0.01	<0.01	NS	NS	<0.01	<0.01
	Group 3 *versus* 4	<0.05	NS	<0.05	NS	<0.01	NS

NS = not significant, $P > 0.05$.

Discussion

It appears that sodium oleate infused intravenously can suppress the volume of gastric secretion collected during 24 hour infusion period. It affects both nonstimulated gastric secretion and histamine stimulated secretion. It is known that spontaneous gastric secretion in rats, or so called 'interdigestive phase' is vagus dependent and represents a true cephalic phase (Komarov *et al.*, 1944; Lin and Alphin, 1958). Under our experimental conditions of the collection of gastric juice, this 'interdigestive phase' is identical with nonstimulated secretion of saline infused rats. Our not yet published studies have shown, that bilateral vagotomy completely suppresses the secretion of HCl in rats, infused for 24 h with normal saline. It may be stated therefore, that the infusion of sodium oleate depressed the volume of gastric juice during the interdigestive phase of gastric secretory function. It also significantly affected histamine stimulated gastric secretion. In this study we used maximal stimulation with histamine, which is known to increase volume of secretion, and concentration of HCl in fasting rats bearing gastric fistulas. In the presence of sodium oleate histamine did not produce such a stimulating effect on gastric mucosa. It is possible that sodium oleate altered reactivity of gastric glandular mucosa to the histamine stimulation. We observed similar change in the reactivity to histamine in our previous study of gastric secretion in rats fed with fat rich diet (Kowalewski, 1969).

In the present study the effect of sodium oleate on gastric secretion is not dependent on the contact of this fatty acid with the duodenal mucosa, but appears to be a function of its presence in the bloodstream. Our work confirms and extends the results of Rochman *et al.* (1969) and demonstrates again that the increase of some fatty acids in the blood may influence secretory function of rat's stomach. It may inhibit the secretion of cephalic phase of gastric secretion in this animal. It may also suppress the histamine stimulated gastric secretory function.

Summary

Intravenous infusion of sodium oleate into rats bearing permanent gastric and vascular cannulas was performed for 24 h. Gastric secretion was collected during the perfusion period. The

effect of sodium oleate was studied during the interdigestive cephalic phase of gastric secretion or during the infusion of maximal dose of histamine. In both instances, sodium oleate suppressed gastric secretory function. It appears from this study, that the contact of a fatty acid with duodenal mucosa is not a necessary condition for the suppression of gastric secretory function by the fat. Increased amount of a fatty acid in the bloodstream may induce the inhibition of gastric secretory function in rats, even in the presence of maximal stimulation with histamine.

Acknowledgments

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The role of lipids in determining certain vitamin requirements

by

Raoul Lecoq, Paul Chauchard and Henriette Mazoué.

The role of vitamins in the organism has become increasingly well known, notably the fact that they play a role in the synthesis of enzymes that are essential for the metabolism of organic and mineral substances. The role of the B vitamins in carbohydrate metabolism is a fact which has long been known; but their action is not limited to this, and Lecoq has shown that at least some of them are also essential to a lesser degree in protein and lipid metabolism (1). So far as proteins are concerned, we have been able to characterize a number of vitamins by means of the chronaximetric method, the need for which is linked to the presence of certain amino acids in the diet or the organism (2). We decided also to investigate those that play a role in the metabolism of lipids. This work was based on recent investigations by Champougny and Le Breton, who have shown that nicotinic acid amide, pantothenic acid and adermine (or pyridoxine) serve as coenzymes for the dehydrogenase of higher fatty acids (3).

Using rats, we first of all investigated the influence of subcutaneous injections of certain lipids (linseed oil, olive oil and castor oil) and fatty acids salts (oleate, stearate and sodium linoleate). While the injection of 1/2 or 1 cc olive oil or linseed oil has no effect, the injection of castor oil gives

rise to a rapid drop in nerve chronaxy corresponding to an encephalomedullary stimulating action (persisting under anesthesia). Muscular chronaxy remains unchanged. The same reactions are observed after the injection of a solution of oleate, stearate or sodium linoleate.

By repeating the olive oil or linseed oil injections daily, there is no change, even after one week, in the neuromuscular excitability. On the other hand castor oil and fatty acid salts lastingly reduce nerve chronaxy, and somewhat later, they increase muscular chronaxy; here again the nervous excitation is of encephalomedullary origin.

Castor oil, whose unbalancing effect linked to its special solubility has been established by Lecoq and Savarre (4), is thus similar to the sodium salts of fatty acids which also have an unbalancing effect because of their speed of absorption or diffusion, while olive oil and linseed oil behave like balancing lipids. Under the same conditions codliver oil gives rise to the reverse neuromuscular disturbances, probably due to its vitamin D content.

Investigating the effect of various vitamins on chronaxial disturbances produced by injections of castor oil, oleate, stearate or linoleate, it was noted that the absorption of thiamine, riboflavin, adenine, paraaminobenzoic acid, folic acid and axerophthol has no effect, while the ingestion of nicotinamide, pantothenic acid and adermine (administered daily in doses of 100-200 γ) preventively or curatively hinders the neuromuscular excitability referred to above.

In conclusion, it can be seen that the metabolism of lipids and fatty acids (which depends on the speed of their absorption and diffusion) determines the need for nicotinamide, pantothenic acid or adermine, these principal vitamins being interchangeable. Thus the fingins of Champougny and Le Breton on the process of apodehydrogenase of fatty acids extracted from the mammalian liver tissue have been confirmed and expanded.

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See footnote references.

Le rôle des lipides dans le conditionnement de certains besoins vitaminiques.

par Raoul Lecoq, Paul Chauchard et Henriette Mazoué.

Le rôle des vitamines dans l'organisme est aujourd'hui de mieux en mieux connu et l'on sait que celles-ci interviennent notamment dans la synthèse de ferments indispensables au métabolisme des éléments organiques et minéraux. L'intervention des vitamines B dans le métabolisme des glucides est un fait admis depuis longtemps ; mais là ne se borne pas leur action et Lecoq a prouvé qu'au moins certaines d'entre elles sont également indispensables (pour une part moins importante) dans le métabolisme des protides et des lipides (1*). En ce qui concerne les protides, nous avons déjà pu caractériser, grâce à la méthode chronaximétrique, une série de vitamines dont le besoin est lié à la présence, dans la ration ou dans l'organisme, de certains acides aminés (2*). Nous nous sommes proposé de rechercher également celles qui interviennent dans le métabolisme des lipides. Pour cela, nous avons pris comme base les récents travaux de Champougny et Le Breton qui ont montré que l'amide nicotinique, l'acide pantothénique et l'adermine (ou pyridoxine) servent de coenzymes à la déhydrogénase des acides gras supérieurs (3*).

Nous avons tout d'abord recherché sur le Rat, l'influence qu'exerce l'injection sous-cutanée de quelques lipides (huiles de lin, d'olive et de ricin) et de quelques sels d'acides gras (oléate, stéarate et linoléate de sodium). Tandis que l'injection de 1/2 ou 1 cm³ d'huile d'olive ou de lin reste sans effet, l'injection d'huile de ricin entraîne une rapide chute des chronaxies nerveuses correspondant à une action excitante encéphalomédullaire (persistance sous anesthésie), les chro-

(1*) *Bull. Soc. Chim. biol.*, 1932, t. *14*, p. 1067 et 1933, t. *15*, p. 1498.
(2*) *C. R. de la Soc. de biol.*, 1947, t. *141*, p. 220.
(3*) *C. R. de la Soc. de biol.*, 1947, t. *141*, p. 43 et 45.

naxies musculaires restent inchangées. Les mêmes réactions s'observent après injection d'une solution d'oléate, de stéarate ou de linoléate de sodium.

En répétant chaque jour les injections d'huile d'olive ou de lin, on n'a, même au bout d'une semaine, aucune modification de l'excitabilité neuromusculaire. Au contraire l'huile de ricin et les sels d'acides gras diminuent de façon durable les chronaxies nerveuses et, un peu plus tardivement, augmentent les chronaxies musculaires ; ici encore l'excitation nerveuse est d'origine encéphalomédullaire.

L'huile de ricin, dont Lecoq et Savare ont établi l'action déséquilibrante (4*) liée à sa particulière solubilité, rejoint ainsi les sels sodiques d'acides gras également déséquilibrants en raison de leur rapidité d'absorption ou de diffusion, alors que les huiles d'olive et de lin se comportent comme des lipides d'équilibre. Dans les mêmes conditions, l'huile de foie de morue provoque des troubles neuromusculaires inverses, vraisemblablement liés à sa teneur en vitamine D.

Recherchant l'action de diverses vitamines sur les perturbations chronaxiques produites par les injections d'huile de ricin, d'oléate, de stéarate ou de linoléate, nous avons noté que l'absorption d'aneurine, de riboflavine, d'adénine, d'acide paraaminobenzoïque, d'acide folique et d'axérophtol demeurent sans influence, tandis que l'ingestion de nicotamide, d'acide pantothénique ou d'adermine (ces substances étant données quotidiennement à raison de 100 à 200 γ) empêchent préventivement ou curativement les troubles de l'excitabilité neuromusculaire précédemment enregistrées.

On voit, en conclusion, que la métabolisation des lipides et des sels d'acides gras (qui est sous la dépendance de leur rapidité d'absorption et de diffusion) conditionne le besoin de nicotamide, d'acide pantothénique ou d'adermine, ces principes vitaminiques pouvant se substituer indifféremment l'un à l'autre. Ainsi se trouvent confirmées et étendues les observations faites par Champougny et Le Breton sur le processus de l'anodéhydrogénase des acides gras extraite des tissus hépatiques des Mammifères.

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CHAPTER 19 OXIDATION OF FATTY ACIDS

Although carbohydrates, because of their abundance, are the major fuel for most organisms, fatty acids also play an extremely important role as energy source. Fatty acid oxidation is prominent in higher animals and plants, which can store large amounts of neutral fat as fuel reserve. Neutral fat has a high caloric value (~9 kcal $gram^{-1}$) and can be stored in nearly anhydrous form in intracellular fat droplets, whereas glycogen or starch (caloric value ~4 kcal $gram^{-1}$) are too hydrated to be stored in such concentrated form. In vertebrates, it is estimated that fatty acid oxidation normally provides at least half of the oxidative energy in the liver, kidneys, heart muscle, and resting skeletal muscle. In fasting, or in hibernating animals or migrating birds, fat is virtually the sole source of energy. On the other hand, little or no fatty acid oxidation occurs in the brain, which depends solely on glucose as energy source.

Intracellular Hydrolysis of Lipids

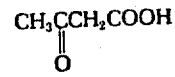
Fatty acids undergoing oxidation in tissues of higher animals come either from extracellular fluid or from endogenous intracellular lipids. Vertebrate blood contains considerable amounts of triacylglycerols and phosphoglycerides and very small amounts of free fatty acids noncovalently bound to the protein serum albumin, which serves to transport fatty acids. The latter are directly oxidized in such tissues as heart and muscle. The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consist largely of triacylglycerols. Since fatty acids must be in free form, i.e., non-esterified, before they can undergo activation and oxidation, the triacylglycerols must first undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerol (Chapter 10). Another source of fatty acids are the membrane phosphoglycerides (Chapter 10). Although they are relatively constant in composition and amount, because of their structural role, they undergo continuous metabolic turnover in higher animal cells (Chapter 13) and yield free fatty acids following action of phospholipases (Chapter 10).

Relatively little is known about the sequence and details of intracellular lipid hydrolysis. However, normally there is no significant accumulation of free fatty acids or other hydrolysis products, such as lysophosphatides, which are extremely toxic and injurious to membrane structure. Evidently the rate and pathway of the hydrolysis of intracellular lipids is adjusted to the rate of utilization of fatty acids.

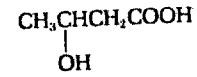
The Fatty Acid Oxidation Cycle

It was long suspected that fatty acids are synthesized and degraded in the cell by addition or subtraction of two-carbon fragments because most natural fatty acids have an even number of carbon atoms. This idea was also supported by the observation that in fasting mammals or in the disease diabetes mellitus fatty acids are incompletely oxidized, leading to the accumulation of the β-oxidized four-carbon acids *acetoacetic acid* and D-*β-hydroxybutyric acid* (margin) in the blood and urine. Another line of evidence came from classical experiments by the German biochemist Knoop in 1904, who observed that rabbits fed an even-carbon fatty acid labeled with a phenyl group at the terminal, or ω-carbon, atom excreted urinary *phenylacetic acid* (Figure 19-1), regardless of the length of the carbon chain of the acid. On the other hand, when ω-phenyl derivatives of odd-carbon fatty acids were fed, *benzoic acid* was excreted. (Phenylacetic and benzoic acid appear in the urine in conjugated form, esterified with D-glucuronic acid.)

Acetoacetic acid



D-β-Hydroxybutyric acid



These results suggested that ω-phenyl fatty acids are degraded by removal of successive two-carbon fragments starting from the carboxyl end. In the even-carbon acids, the last two-carbon fragment is not oxidized further but is excreted as phenylacetic acid. Successive removal of two-carbon fragments from odd-carbon acids would yield the phenyl-substituted terminal carbon atom, which is excreted as benzoic acid. From these experiments (perhaps the first metabolic tracer experiments reported) Knoop postulated that fatty acids are oxidized by *β oxidation*, i.e., oxidation at the β carbon to yield a β-keto acid, which was assumed to undergo cleavage to form acetic acid and a fatty acid shorter by two carbon atoms.

For many years, however, no success attended efforts to demonstrate fatty acid oxidation in cell-free extracts of animal tissues known to be active in this process. The enzymes involved in oxidation of fatty acids were believed to require intact cell structure. Furthermore, no evidence could be found of the occurrence of the medium-length β-keto fatty acid intermediates predicted by the β-oxidation hypothesis. Not until 1943 was oxidation of fatty acids by molecular oxygen in a cell-free system first demonstrated, by Leloir in Argentina, who found the system to be extremely labile. Shortly later, Lehninger showed that when ATP was added to homogenates of rat liver, the ability to oxidize fatty acids was restored, and he postulated that ATP was required to activate the fatty acid enzymatically. He also found that fatty acids are degraded into two-carbon units that can enter the Krebs

Figure 19-1
Knoop's experiments on oxidation of phenyl fatty acids. Even-carbon acids always yield phenylacetic acid; odd-carbon acids always yield benzoic acid.

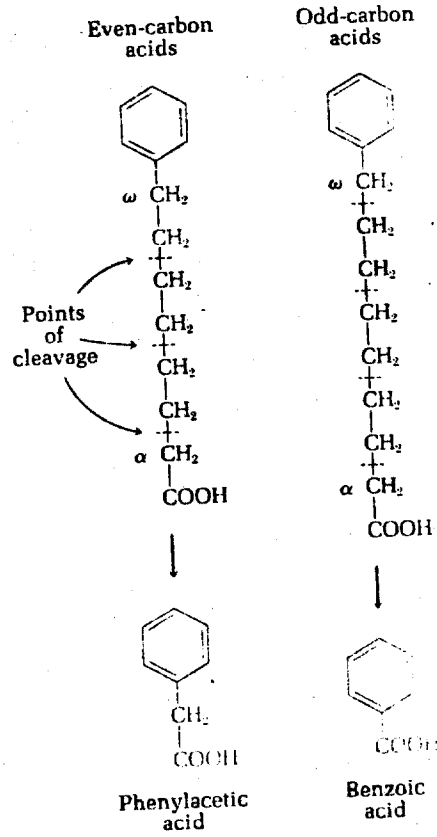
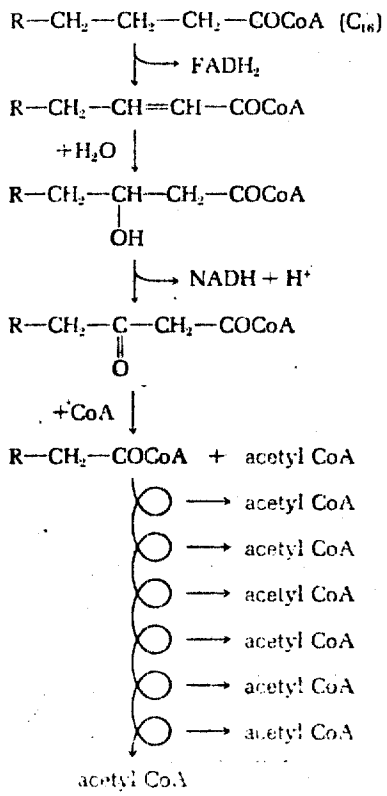


Figure 19-2
The fatty acid oxidation spiral. One acetyl CoA is removed during each pass through the sequence. One molecule of palmitic acid (C_{16}), after activation to palmitoyl CoA, yields eight molecules of acetyl CoA.



tricarboxylic acid cycle. In 1948–1949, Kennedy and Lehninger showed that the oxidation of fatty acids occurs exclusively in the mitochondria.

The next important clue, which quickly led to recognition of the nature of the enzymatic steps of fatty acid oxidation, came from work of Lynen and his colleagues in Germany. They found that the ATP-dependent activation of fatty acids involves their esterification with the thiol group of CoA and that all the subsequent enzymatic steps in fatty acid oxidation take place in the form of their CoA esters. This observation explained why intermediate-length free fatty acids or β-keto acids are not found in most cells and tissues during oxidation of long-chain fatty acids. The various enzymes catalyzing the successive steps in fatty acid oxidation have since been isolated in highly purified form by Lynen, Green, Ochoa, and other investigators. The overall pathway of fatty acid oxidation is shown in Figure 19-2. Long-chain fatty acids are first activated by an energy-requiring step in which the fatty acid ester of CoA is formed enzymatically at the expense of ATP. This step is analogous to the priming step of glycolysis, in which glucose is phosphorylated by ATP. The fatty acids then enter the mitochondria as esters of the compound *carnitine* and are converted back into CoA esters. In the first step of the oxidative cycle, the fatty acyl CoA is dehydrogenated by removal of a pair of hydrogen atoms from the α and β carbon atoms to yield the α,β-unsaturated acyl CoA. The latter is then enzymatically hydrated to form a β-hydroxyacyl CoA ester, which in turn is dehydrogenated in an NAD-linked reaction to form the β-ketoacyl CoA ester. The latter then undergoes enzymatic cleavage by attack of the thiol group of a second molecule of CoA, to form acetyl CoA, derived from the α and the carboxyl carbon atoms of the original fatty acid chain. The other product, a long-chain saturated fatty acyl CoA ester having two fewer carbon atoms than the starting fatty acid, now becomes the substrate for another round of reactions, beginning with the first dehydrogenation step, until a second two-carbon fragment is removed as acetyl CoA. At each passage through this spiral process, the fatty acid chain loses a two-carbon fragment as acetyl CoA and two pairs of hydrogen atoms to specific acceptors. The 16-carbon palmitic acid thus undergoes a total of seven such cycles, to yield altogether eight molecules of acetyl CoA and 14 pairs of hydrogen atoms. The palmitic acid must be primed or activated only once, since at the end of each round the shortened fatty acid appears as its CoA ester.

The acetyl CoA formed as product of the fatty acid oxidation system then may enter the tricarboxylic acid cycle. The 14 pairs of hydrogen atoms removed during dehydrogenation of palmitic acid enter the respiratory chain, 7 pairs in the form of the reduced flavin coenzyme of fatty acyl CoA dehydrogenase and 7 pairs in the form of NADH. The passage of electrons from $FADH_2$ to oxygen and from NADH to oxygen in each case leads to the expected number of oxidative phosphorylations of ADP.

During fatty acid oxidation, there is no significant accumulation of the various intermediate CoA esters. The total amount of CoA in mitochondria is in fact very small,

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and fatty acid oxidation cannot continue without regeneration of free CoA, which occurs when acetyl CoA combines with oxaloacetate on entering the tricarboxylic acid cycle. The separate enzymatic steps in fatty acid oxidation will now be examined in detail.

Activation and Entry of Fatty Acids into Mitochondria

Recent research shows that there are three stages in the entry of fatty acids into mitochondria from the extramitochondrial cytoplasm. (1) The enzymatic esterification of the free fatty acid with extramitochondrial CoA, at the expense of ATP, which occurs in the outer membrane (2) the transfer of the fatty acyl group from CoA to the carrier molecule carnitine, which carries it across the inner membrane and (3) the transfer of the fatty acyl group from carnitine to intramitochondrial CoA.

Activation of Fatty Acids

At least three different enzymes catalyze formation of fatty acyl CoA esters, each being specific for a given range of fatty acid chain length. They are called activating enzymes or, better, *fatty acid thiokinases* [International designation *fatty acid:CoA ligase (AMP)*]. *Acetate thiokinase* activates acetic, propionic, and acrylic acids, *medium-chain fatty acid thiokinase* activates fatty acids with 4 to 12 carbon atoms, and *long-chain fatty acid thiokinase* activates fatty acids with 12 to 22 or more carbon atoms. The last two thiokinases activate both saturated and unsaturated fatty acids and α- and β-hydroxy acids. Otherwise the properties and mechanisms of all three thiokinases, which have been isolated in highly purified form, are nearly identical. The overall reaction catalyzed by the ATP-linked thiokinases is

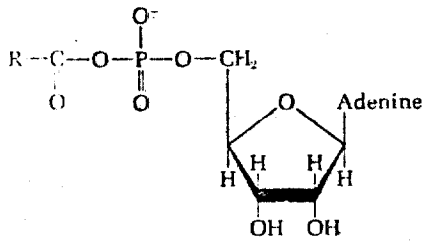
$$\mathrm{RCOOH + ATP + CoASH \rightleftharpoons R{-}\underset{\underset{\mathrm{O}}{\|}}{C}{-}SCoA + AMP + PP_i}$$

As the thioester linkage is formed between the fatty acid carboxyl group and the thiol group of CoA, the ATP undergoes pyrophosphate cleavage (Chapter 14) to yield AMP and inorganic pyrophosphate. The equilibrium constant for fatty acid activation, regardless of chain length, is about 1.0. The standard free energy of hydrolysis of the thioester group of the fatty acyl CoA formed is about the same as that of acetyl CoA ($\Delta G^{\circ\prime} = -7.50$ kcal). The pyrophosphate formed in the activation reaction is hydrolyzed by inorganic pyrophosphatase

$$\mathrm{PP_i + H_2O \longrightarrow 2P_i}$$

The net effect of this hydrolysis is the utilization of two high-energy phosphate bonds to pull the overall equilibrium of the preceding activation step far in the direction of the formation of fatty acyl CoA. Because fluoride, an inhibitor of inorganic pyrophosphatase, inhibits oxidation of free fatty acids, the hydrolysis of pyrophosphate appears to be essential in the overall activation process.

A fatty acyl adenylate

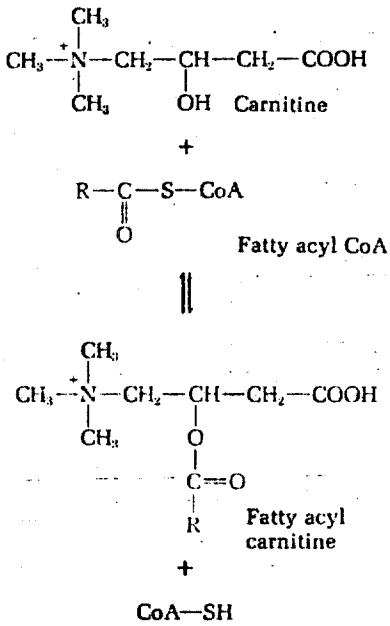


An enzyme-bound intermediate has been identified in the action of fatty acid thiokinases. It is a mixed anhydride of the fatty acid and the phosphate group of AMP, a *fatty acyl adenylate*. It is formed on the active site, with discharge of pyrophosphate to the medium (margin). In the presence of free CoA—SH, the fatty acyl adenylate reacts to yield fatty acyl CoA and free AMP as products.

The fatty acid thiokinases are found in the outer mitochondrial membrane; they are also present in microsomes derived from endoplasmic reticulum.

Transfer to Carnitine

Fatty acyl CoA: carnitine fatty acid transferase reaction



Higher fatty acids have only a limited ability to cross the inner membrane as CoA esters, but their entry is greatly stimulated by *carnitine*, a substance long known to be present in animal tissues but whose function went unrecognized until it was found to be an essential growth factor for the mealworm, *Tenebrio molitor*, the first suggestion that it may be required for some vital cellular function. Later, Fritz and other investigators showed that carnitine has a stimulating effect on the oxidation of fatty acids by mitochondria. It is now known that this effect is due to the action of an enzyme, *fatty acyl CoA:carnitine fatty acid transferase*, which catalyzes transfer of the fatty acyl group from its thioester linkage with CoA to an oxygen-ester linkage with the hydroxyl group of carnitine (margin). Since the standard free-energy change of this reaction is quite small, the acyl carnitine linkage evidently represents a high-energy bond.

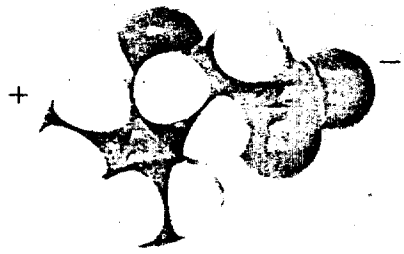
The O-fatty acyl carnitine so formed readily passes through the inner membrane; whether this occurs by simple diffusion or through some membrane carrier mechanism (Chapter 27) is not known.

Transfer to Intramitochondrial CoA

In the last stage of the entry process, the fatty acyl group is transferred from carnitine to intramitochondrial CoA by the action of intramitochondrial carnitine fatty acid transferase

$$\text{Fatty acyl carnitine} + \text{CoA} \rightleftharpoons \text{fatty acyl CoA} + \text{carnitine}$$

Space-filling model of carnitine



This entry mechanism has the effect of keeping extramitochondrial and intramitochondrial CoA separate. The fatty acyl CoA now is used as substrate by the fatty acid oxidation cycle, which occurs in the inner matrix compartment.

Mitochondria contain another type of acyl thiokinase participating in fatty acid activation. Since this enzyme requires GTP instead of ATP and causes an *orthophosphate* cleavage of the GTP, it is systematically named *fatty acid:CoA ligase* (GDP). The overall reaction catalyzed is

$$\text{GTP} + \text{RCOOH} + \text{CoASH} \rightleftharpoons \text{R—C(=O)—SCoA} + \text{GDP} + \text{P}_i$$

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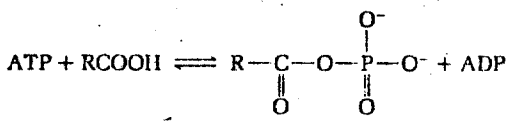
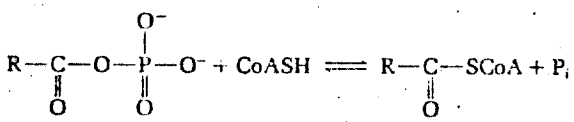
Its equilibrium constant is also approximately 1.0. Presumably the function of this enzyme, which is located in the matrix, is to make possible direct utilization of the GTP generated in mitochondria during the substrate-level phosphorylation associated with oxidation of α-ketoglutarate (Chapter 16). Since carnitine is not necessary for its action, it is probably concerned with the activation of free fatty acids formed internally in mitochondria.

In some microorganisms which ferment fatty acids, e.g., the strict anaerobe *Clostridium kluyveri*, the formation of acyl CoA esters occurs by an indirect route. In the first step a high-energy acyl phosphate of acetate or fatty acid is formed by direct phosphorylation of the carboxyl group by ATP



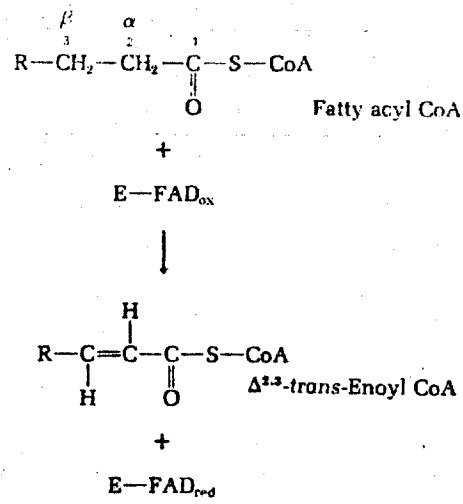
The acyl phosphate so formed, a mixed anhydride of a carboxylic acid and phosphoric acid, is analogous to the 1,3-diphosphoglycerate formed during glycolysis (Chapters 14 and 15). The reaction written above is endergonic, but it is pulled to the right because the acyl phosphate undergoes a rapid reaction with CoA—SH catalyzed by a second enzyme, *phosphotransacetylase*, to form acyl CoA



The First Dehydrogenation Step of Fatty Acid Oxidation

Following the activation step, the reactions of the fatty acid oxidation cycle take place in the inner compartment of mitochondria. The fatty acyl CoA ester first undergoes enzymatic dehydrogenation at the α and β carbon atoms, i.e., atoms 2 and 3, to form an unsaturated fatty acyl CoA as product; the position of the double bond is designated by the symbol $\Delta^{2,3}$. There are four different FAD-containing *fatty acyl CoA dehydrogenases*, each specific for a given range of fatty acid chain length. The reaction catalyzed is shown in the margin.

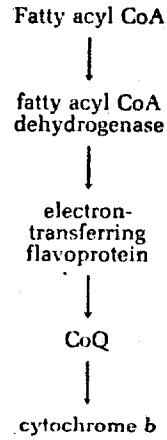
The fatty acyl CoA dehydrogenase reaction.



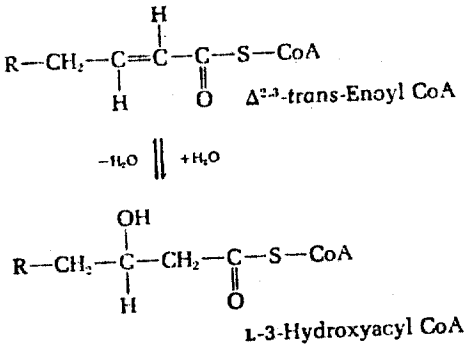
The tightly bound FAD of the enzyme becomes reduced, a process that probably occurs through distinct one-electron steps (Chapter 17). The $\Delta^{2,3}$-unsaturated bond formed in this reaction is the *trans* geometrical isomer. In contrast, the double bonds of the unsaturated fatty acids of natural fats are nearly always in the *cis* configuration (Chapter 10). We shall come back to this interesting point later.

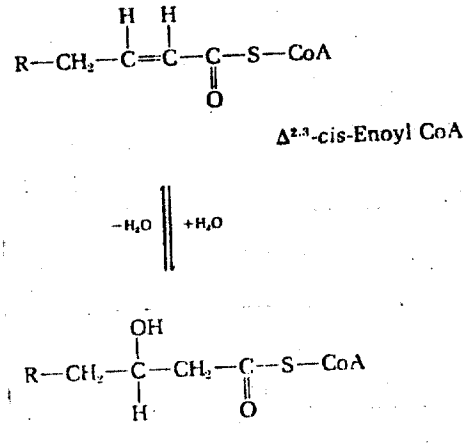
The reduced form of the fatty acyl CoA dehydrogenase cannot react directly with oxygen. It donates its electrons to the respiratory chain via a second flavoprotein, *electron-transferring flavoprotein*, which in turn reduces some electron carrier of the respiratory chain that has not yet been identified with certainty, but is probably Coenzyme Q (margin).

Electron transport from fatty acyl CoA to cytochrome *b*.



The enoylhydratase reactions



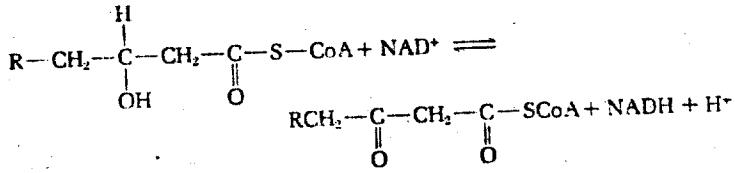


The Hydration Step

The reversible hydration of the double bond of $\Delta^{2,3}$-enoyl CoA esters to form β-hydroxy (or 3-hydroxy) acyl CoA is catalyzed by the enzyme *enoylhydratase* (systematic name, *3-hydroxyacyl CoA hydrolyase*), which has been obtained in crystalline form. The reaction catalyzed is shown in the margin. The addition of water across the $\Delta^{2,3}$-*trans* double bond is stereospecific and results in the formation of the L stereoisomer of the 3-hydroxyacyl CoA ester. Enoylhydratase cannot hydrate $\Delta^{3,4}$-unsaturated fatty acyl CoA esters, but it can hydrate $\Delta^{2,3}$-*cis*-unsaturated fatty acyl CoA esters to yield the corresponding D stereoisomer of 3-hydroxy fatty acyl CoA esters (margin). This may appear puzzling, since the next enzyme in the fatty acid oxidation cycle is specific for the L stereoisomer of 3-hydroxy fatty acyl CoA. We shall see later, however, that the D- and L- stereoisomers of 3-hydroxy fatty acyl CoA differ in origin and function.

The Second Dehydrogenation Step

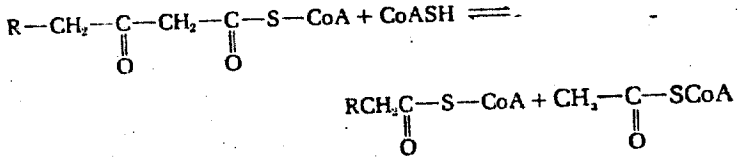
In the next step of the fatty acid cycle, the L-3-hydroxy fatty acyl CoA is dehydrogenated to form 3-keto fatty acyl CoA by *L-3-hydroxy fatty acyl CoA dehydrogenase;* NAD^+ is the specific electron acceptor. The reaction is



This enzyme is relatively nonspecific for fatty acid chain length but absolutely specific for the L stereoisomer. The NADH formed in the reaction donates its electron equivalents to the NADH dehydrogenase of the respiratory chain.

The Thiolytic Cleavage Step

In the last step of the fatty acid oxidation sequence, which is catalyzed by *β-ketothiolase* (more simply, *thiolase*), the 3-keto fatty acyl CoA undergoes cleavage by interaction with a molecule of free CoA, to yield the carboxyl-terminal two-carbon fragment as free acetyl CoA and the CoA ester of a fatty acid shortened by two carbon atoms



This cleavage reaction, also called a *thiolysis* or a *thiolytic cleavage*, is analogous to a hydrolysis. The reaction is highly exergonic and thus the equilibrium favors cleavage. There appear to be two or three forms of the enzyme, each specific for different chain lengths.

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The Balance Sheet

With the thiolysis reaction we have completed one round of the fatty acid oxidation sequence, in which one molecule of acetyl CoA and two pairs of hydrogen atoms have been removed from a long-chain fatty acyl CoA. The overall equation for one turn of the fatty acid oxidation sequence acting on palmitoyl CoA is

$$\text{Palmitoyl CoA} + \text{CoA} + \text{FAD} + \text{NAD}^+ + \text{H}_2\text{O} \longrightarrow \text{myristoyl CoA} + \text{acetyl CoA} + \text{FADH}_2 + \text{NADH} + \text{H}^+$$

We can now write the equation for the seven turns of the spiral required to convert one molecule of palmitoyl CoA to eight molecules of acetyl CoA

$$\text{Palmitoyl CoA} + 7\text{CoA} + 7\text{FAD} + 7\text{NAD}^+ + 7\text{H}_2\text{O} \longrightarrow 8\ \text{acetyl CoA} + 7\text{FADH}_2 + 7\text{NADH} + 7\text{H}^+$$

Each molecule of reduced fatty acyl CoA dehydrogenase ($FADH_2$) donates a pair of electron equivalents to the respiratory chain at the level of CoQ or cytochrome b, and two molecules of ATP are generated during the ensuing electron transport (Chapter 17). Similarly, oxidation of each molecule of NADH results in formation of three molecules of ATP. Hence, a total of five molecules of ATP is formed per molecule of acetyl CoA cleaved. We can therefore write the following equation, which includes the phosphorylations:

$$\text{Palmitoyl CoA} + 7\text{CoA} + 7\text{O}_2 + 35\text{P}_i + 35\text{ADP} \longrightarrow 8\ \text{acetyl CoA} + 35\text{ATP} + 42\text{H}_2\text{O} \quad (1)$$

The eight molecules of acetyl CoA formed in the fatty acid cycle may now enter the Krebs cycle. The following equation represents the balance sheet for their oxidation and the coupled phosphorylations:

$$8\ \text{acetyl CoA} + 16\text{O}_2 + 96\text{P}_i + 96\text{ADP} \longrightarrow 8\text{CoA} + 96\text{ATP} + 104\text{H}_2\text{O} + 16\text{CO}_2 \quad (2)$$

Combining equations (1) and (2), we get the overall equation

$$\text{Palmitoyl CoA} + 23\text{O}_2 + 131\text{P}_i + 131\text{ADP} \longrightarrow \text{CoA} + 16\text{CO}_2 + 146\text{H}_2\text{O} + 131\text{ATP}$$

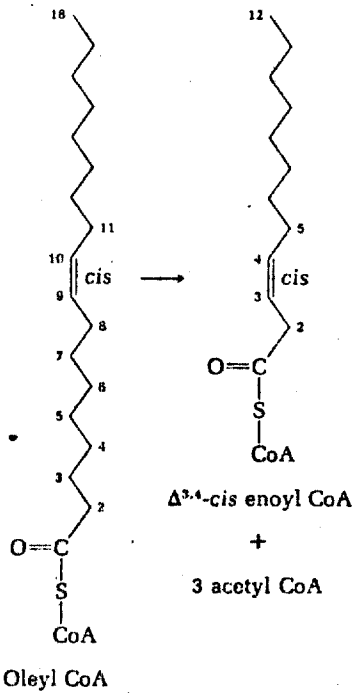
Since one molecule of ATP (or GTP) was required to activate the free palmitic acid to begin with, we may assume the net yield of ATP to be 130 molecules and write the partial equations for the exergonic and endergonic processes

$$\text{Palmitic acid} + 23\text{O}_2 \longrightarrow 16\text{CO}_2 + 16\text{H}_2\text{O} \qquad \Delta G^{\circ\prime} = -2{,}340\ \text{kcal}$$

$$130\text{ADP} + 130\text{P}_i \longrightarrow 130\text{ATP} + 130\text{H}_2\text{O} \qquad \Delta G^{\circ\prime} = 130 \times 7.30 = +949\ \text{kcal}$$

Thus some $949/2{,}340 \times 100 = 40$ percent of the standard free energy of oxidation of palmitic acid is recovered as phosphate-bond energy.

Oxidative removal of 3 acetyl CoA units from oleic acid

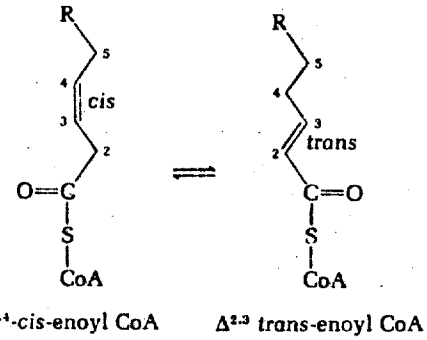


Oxidation of Unsaturated Fatty Acids

Unsaturated fatty acids, such as oleic acid, are oxidized by the same general pathways as saturated fatty acids, but two special problems arise. The double bonds of naturally occurring unsaturated fatty acids are in the *cis* geometrical configuration, whereas the $\Delta^{2,3}$-unsaturated acyl CoA esters functioning as intermediates in the oxidation of saturated fatty acids are *trans*. Moreover, the double bonds of most unsaturated fatty acids occur at such positions in the carbon chain that successive removal of two-carbon fragments from the carboxyl end up to the point of the first double bond yields a $\Delta^{3,4}$-unsaturated fatty acyl CoA rather than the $\Delta^{2,3}$-fatty acyl CoA serving as an intermediate in the fatty acid cycle (shown in margin for oleic acid). However, it will be recalled that enoylhydratase cannot hydrate $\Delta^{3,4}$-unsaturated fatty acyl CoA esters.

This anomaly has recently been resolved with the discovery of an auxiliary enzyme, *$\Delta^{3,4}$cis-$\Delta^{2,3}$-trans-enoyl CoA isomerase*, which catalyzes the reversible shift of the double bond from the $\Delta^{3,4}$-*cis* to the $\Delta^{2,3}$-*trans* configuration. The equilibrium constant of this reaction (margin) is about 7.0. The resulting $\Delta^{2,3}$-*trans*-unsaturated fatty acyl CoA is the normal substrate for the next enzyme of the fatty acid oxidation sequence, *enoyl hydratase*, which hydrates it to form L-3-hydroxy fatty acyl CoA (see above). The complete oxidation of oleyl CoA to nine acetyl CoA units by the fatty acid oxidation cycle thus requires an extra enzymatic step catalyzed by the $\Delta^{3,4}$-*cis*-$\Delta^{2,3}$-*trans* isomerase, in addition to the enzymes required in saturated fatty acid oxidation.

$\Delta^{3,4}$ *cis*-$\Delta^{2,3}$ *trans*-enoyl CoA isomerase reaction



Polyunsaturated fatty acids, such as linoleic acid (Chapter 10), require a second auxiliary enzyme to complete their oxidation, since they contain two or more *cis* double bonds. When three successive acetyl CoA units are removed from linoleyl CoA, a $\Delta^{3,4}$-*cis* double bond is reached, as in the case of oleyl CoA. This is transformed by the $\Delta^{3,4}$-*cis*-$\Delta^{2,3}$-*trans* isomerase to the $\Delta^{2,3}$-*trans* isomer, which is then hydrated to the corresponding L-3-hydroxy fatty acyl CoA, dehydrogenated, and cleaved by thiolase to yield acetyl CoA and an eight-carbon $\Delta^{2,3}$-unsaturated acid (Figure 19-3); however, the double bond of the latter is in the *cis* configuration. Although the $\Delta^{2,3}$-*cis* double bond can be hydrated by enoyl hydratase, as mentioned above, the product is the D stereoisomer of a 3-hydroxy fatty acyl CoA, not the L stereoisomer formed during oxidation of saturated fatty acids. The utilization of the D-stereoisomeric 3-hydroxyacyl CoA ester generated from linoleic acid requires a second auxiliary enzyme, *3-hydroxy fatty acyl CoA epimerase*, which catalyzes the epimerization of carbon atom 3 (below). The product of

Conversion of $\Delta^{2,3}$ *cis*-enoyl CoA to L-3-hydroxy acyl CoA.

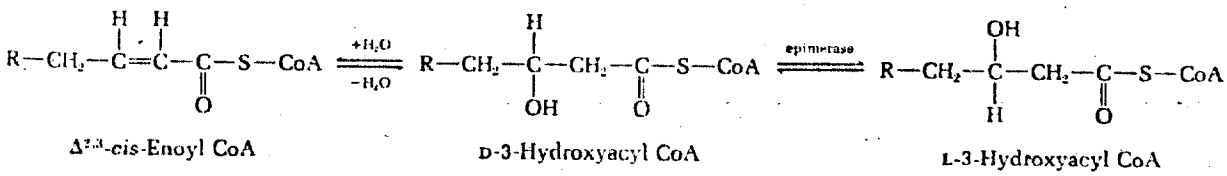
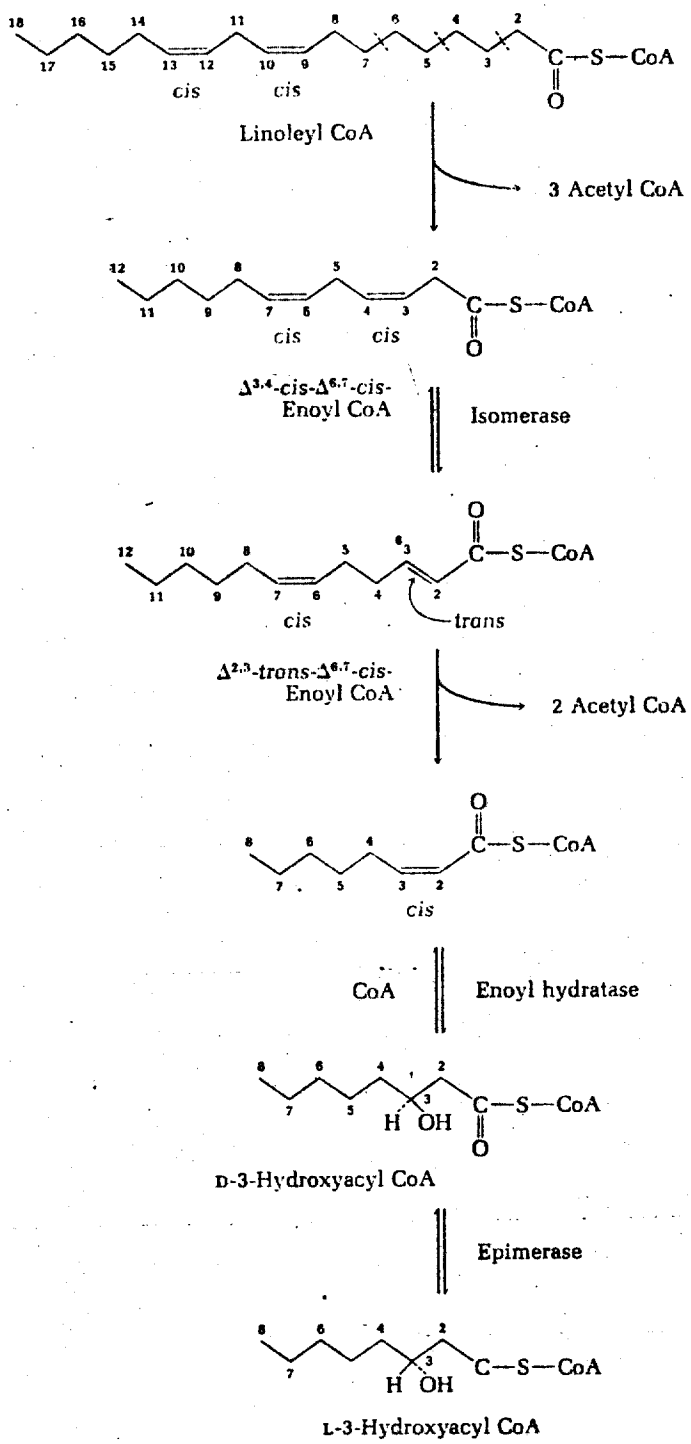


Figure 19-3
Pathway of oxidation of linoleic acid, showing action of auxiliary enzymes.



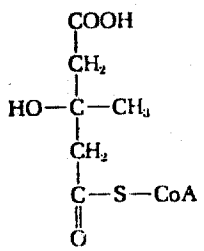
this freely reversible reaction is then oxidized by the L-specific 3-hydroxy fatty acyl CoA dehydrogenase and cleaved by thiolase to complete the oxidation cycle. The remaining six-carbon saturated fatty acyl CoA derived from linoleic acid can now be oxidized to three molecules of acetyl CoA. These two auxiliary enzymes of the fatty acid oxidation cycle make possible the complete oxidation of all the common unsaturated fatty acids found in naturally occurring lipids.

Ketone Bodies and Their Oxidation

In many vertebrates the liver has the enzymatic capacity to divert some of the acetyl CoA derived from fatty acid or pyruvate oxidation, presumably during periods of excess formation, into free acetoacetate and D-β-hydroxybutyrate, which are transported via the blood to the peripheral tissues, where they may be oxidized via the tricarboxylic acid cycle. These compounds are called the *ketone bodies*. Acetoacetate is formed in two ways. Some arises as the CoA ester from the last four carbon atoms of a long-chain fatty acid following prior removal of successive acetyl CoA residues during fatty acid oxidation, but most arises from the condensation of two molecules of acetyl CoA, catalyzed by thiolase

$$\text{Acetyl CoA} + \text{acetyl CoA} \rightleftharpoons \text{acetoacetyl CoA} + \text{CoA}$$

β-Hydroxy-β-methylglutaryl CoA



The acetoacetyl CoA formed in these reactions then undergoes loss of CoA to become acetoacetate, a process known as *deacylation*. The major pathway for deacylation is the formation and enzymatic cleavage of *β-hydroxy-β-methylglutaryl CoA* (margin), which also serves as a precursor of sterols (Chapter 23)

$$\text{Acetoacetyl CoA} + \text{acetyl CoA} \rightleftharpoons \beta\text{-hydroxy-}\beta\text{-methylglutaryl CoA} + \text{CoA}$$

$$\beta\text{-Hydroxy-}\beta\text{-methylglutaryl CoA} \longrightarrow \text{acetoacetate} + \text{acetyl CoA}$$

The sum of these two reactions is

$$\text{Acetoacetyl CoA} \longrightarrow \text{acetoacetate} + \text{CoA}$$

The free acetoacetate so produced is enzymatically reduced to D-β-hydroxybutyrate by the NAD-linked *D-β-hydroxybutyrate dehydrogenase*. This enzyme is specific for the D stereoisomer; it does not act on CoA esters

$$\text{Acetoacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{D-}\beta\text{-hydroxybutyrate} + \text{NAD}^+$$

The mixture of free acetoacetate and β-hydroxybutyrate resulting from this reaction then may diffuse out of the liver cells into the blood stream to the peripheral tissues. Normally the concentration of ketone bodies in the blood is very low, but in fasting or in the disease diabetes mellitus, it may reach extremely high levels. This condition, known as *ketosis*, arises when the rate of formation of the ketone bodies exceeds the capacity of the peripheral tissues to utilize them.

In the peripheral tissues the D-β-hydroxybutyrate is oxidized to acetoacetate, which is then activated to form its CoA ester. Two enzymatic mechanisms for activation of acetoacetate are known. In the first, ATP is utilized in a reaction similar to the activation of fatty acid

$$\text{ATP} + \text{acetoacetate} + \text{CoA} \rightleftharpoons \text{acetoacetyl CoA} + \text{AMP} + \text{PP}_i$$

In the second, CoA is enzymatically transferred from succinyl CoA (Chapter 16) to acetoacetate, with little change in free energy. The acetoacetyl CoA formed in these reactions then undergoes thiolytic cleavage to two molecules of acetyl CoA, which then may enter the tricarboxylic acid cycle.

Activation of acetoacetate

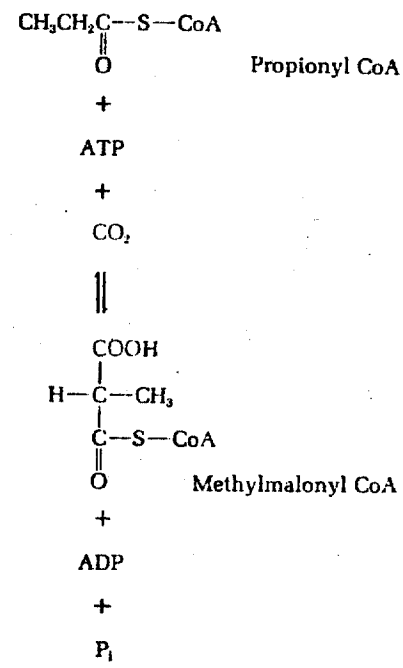
Succinyl CoA
+
acetoacetate
⇌
succinate
+
acetoacetyl CoA

Oxidation of Odd-Carbon Fatty Acids

Odd-carbon fatty acids, which rarely occur in nature but arise during the oxidative degradation of valine and isoleucine (Chapter 20), can also be oxidized in the fatty acid oxidation cycle. Successive acetyl CoA residues are removed until the terminal three-carbon atom residue is reached; it becomes propionyl CoA.

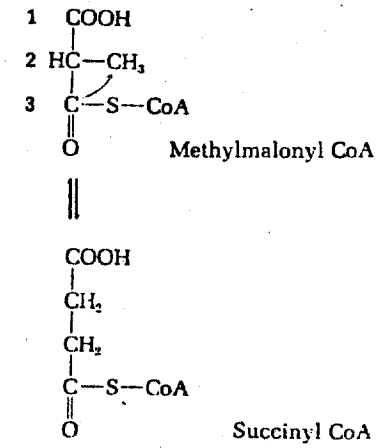
Propionyl CoA undergoes enzymatic carboxylation to form *methylmalonyl CoA* (margin), catalyzed by *propionyl carboxylase*. This enzyme contains biotin as coenzyme (Chapter 16). In the following reaction step, catalyzed by *methylmalonylmutase*, methylmalonyl CoA is isomerized to succinyl CoA (margin) which may undergo deacylation in the succinyl thiokinase reaction to yield free succinate, which may enter the tricarboxylic acid cycle.

Propionylcarboxylase reaction



Although the metabolism of propionyl CoA in animal tissues appears to be a relatively minor pathway, quantitatively speaking, the methylmalonylmutase step involves a coenzyme of extraordinary chemical and biological properties. It is 5′-deoxyadenosylcobalamin, a derivative of *vitamin* B_{12}, which is necessary in the nutrition of man and probably most animal and plant species. The history of vitamin B_{12} is of interest since its discovery, isolation, structure determination, and mode of action have been exceedingly challenging problems. In 1926 Minot and Murphy discovered that eating liver cures patients suffering from pernicious anemia. Despite many attempts over the years to isolate the liver factor, little progress was made, since human pernicious anemia patients had to be used to assay the potency of liver-factor concentrates. In 1948, the liver factor, called vitamin B_{12}, was finally isolated in crystalline form. Despite a wide distribution in nature, the vitamin is present in only the minutest quantities in most organisms. Blood, for example, contains only 2×10^{-4} μg ml^{-1}. The richest sources of vitamin B_{12} are sewage, manure, soil, and mud, which contain anaerobic bacteria rich in the vitamin.

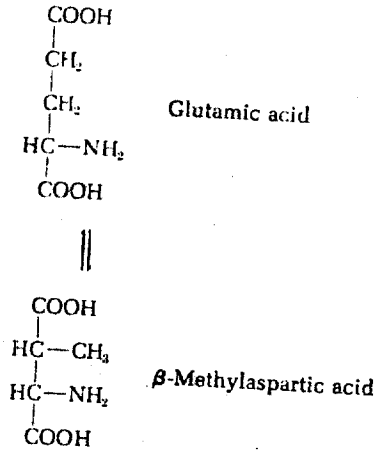
Methylmalonyl mutase reaction



Note that the entire —CO—S—CoA group is transferred from carbon atom 2 to the methyl carbon atom.

Pernicious anemia is not simply a deficiency of vitamin B_{12} but results from the failure to absorb vitamin B_{12} from ingested food, due to a lack of a mucoprotein in gastric juice called the *intrinsic factor*. Vitamin B_{12} promotes the development of normal erythrocytes. However, its mode of action was obscure until 1958, when Barker discovered that the 5′-deoxyadenosyl derivative of vitamin B_{12} is an essential coenzyme in the conversion of glutamic acid into β-methylaspartic acid (margin) in an anaerobic bacterium, *Clostridium tetanomorphum*. It was subsequently discovered that this coenzyme is also required for the action of *methylmalonylmutase*, which converts the car-

Formation of β-methylaspartic acid



boxylation product of propionyl CoA, namely, methylmalonyl CoA, into succinyl CoA.

Elucidating the structure of vitamin B_{12} (Figure 19-4) proved to be an extraordinarily difficult problem, which was solved by a combination of chemical and x-ray crystallography approaches in 1957. Vitamin B_{12} has two characteristic components. The first is a nucleotidelike structure, which is exceptional in containing as base 5,6-dimethylbenzimidazole in α-glycosidic linkage with D-ribose rather than the β linkage present in most nucleotides; it also contains a phosphate group at the 3′ position. The second and most characteristic part of the molecule is the corrin ring system, which resembles porphyrins (Chapter 17) in containing four pyrrole rings but in which two of the pyrroles are joined directly rather than through methene bridges (Figure 19-4). Coordinated to the four inner nitrogen atoms of the corrin ring is an atom of cobalt, long known as an essential trace metal for growth. Vitamin B_{12} is also known as cobalamin. As usually isolated, the sixth coordination position of the Co(II) atom is filled by cyanide, an artifact of isolation; this derivative is called cyanocobalamin.

Vitamin B_{12} has no coenzyme activity itself, but its 5-deoxyadenosyl derivative is the active coenzyme form. The 5-deoxyadenosyl group is attached to the cobalt of vitamin B_{12} only through its methylene group; it replaces the cyanide of cyanocobalamin.

The methylmalonyl CoA mutase reaction has given a clue to the function of its coenzyme. Study of this intramolecular reaction with isotope tracers has re-

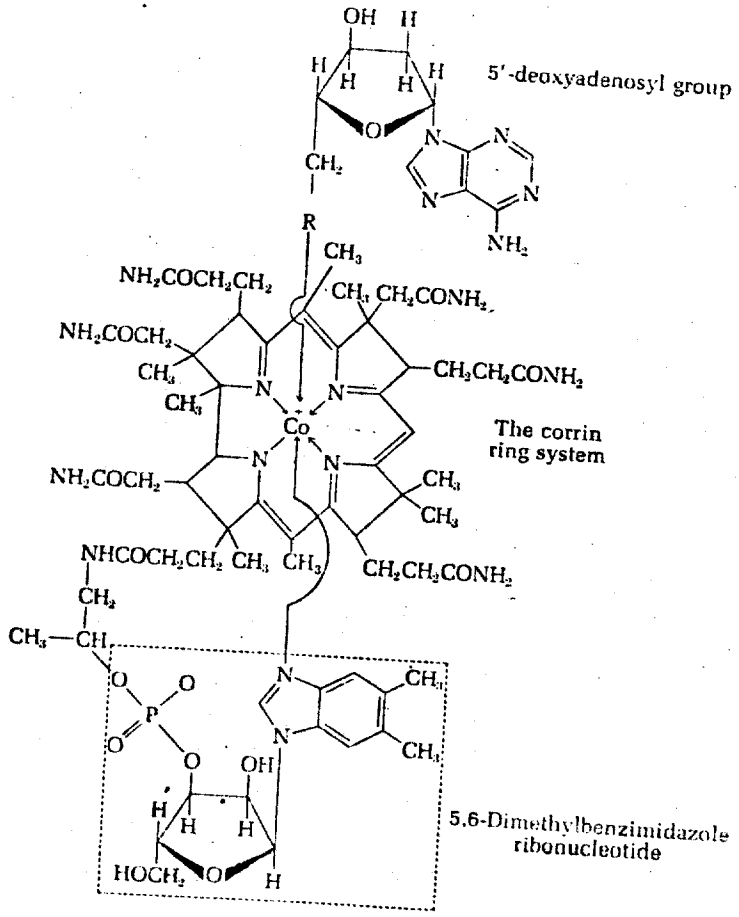


Figure 19-4
Vitamin B_{12} (cobalamin) and its derivatives. In cyanocobalamin R = cyanide; in chlorocobalamin R = chloride. Cobalamin forms similarly named complexes with sulfate, hydroxyl, and nitrite ions. In the coenzyme R is the 5′-deoxyadenosyl group (in color above). The name of the coenzyme is often abbreviated as DAcobalamin.

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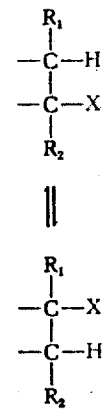
vealed that it takes place by the migration of the entire thioester group (—CO—S—CoA) from carbon atom 2 of methylmalonyl CoA to the methyl carbon atom, in exchange for an H atom. In each of the few known enzymatic reactions in which DAcobalamin is the required coenzyme, the reaction catalyzed is the exchange of a hydrogen atom on one carbon atom with a substituent group on a vicinal carbon atom, without exchange of the hydrogen atom with protons of the medium (margin). The mechanism of this process is not yet understood, but one hypothesis suggests that Co(II) undergoes reduction to Co(I), an extremely strong nucleophile, which can serve as a carrier of alkyl groups from one atom to another. This hypothesis is supported by the fact that this coenzyme is required for the formation of methane gas from methylated compounds in certain anaerobic bacteria and for the transfer of methyl groups in animal tissues (Chapter 24). In fact, an active methyl derivative of vitamin B_{12} has been identified; the methyl group, which replaces the deoxyadenosyl group, is covalently bonded to the cobalt atom. Alkylcobalamins are probably the only organometallic compounds found in nature.

Alkyl group shifts catalyzed by cobamide enzymes



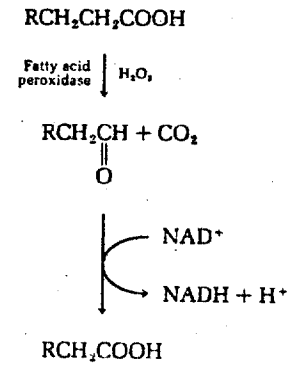
It is noteworthy that people suffering from pernicious anemia, who are deficient in vitamin B_{12} because of the lack of intrinsic factor, excrete large amounts of methylmalonic acid and propionic acid in the urine, proving that the methylmalonylmutase reaction is defective in such patients. The function of the vitamin in transmethylations will be discussed further in Chapters 24 and 25.

Minor Pathways of Fatty Acid Oxidation

The α-hydroxy fatty acids found in some cerebrosides and gangliosides (Chapter 10) are formed by enzymatic hydroxylation of saturated fatty acids. Some fatty acids may also undergo oxidation at the ω carbon atom to form ultimately α,ω-dicarboxylic acids. This pathway is called *ω oxidation*; its function is not known.

In germinating plant seeds a special pathway of fatty acid oxidation occurs, called *α oxidation*, in which the carboxyl carbon is lost as CO_2 and the α carbon is oxidized to an aldehyde group at the expense of hydrogen peroxide. This reaction is catalyzed by *fatty acid peroxidase*. The H_2O_2 required is furnished from other oxidations, presumably of reduced flavoproteins. The fatty aldehyde formed is oxidized to the corresponding carboxylic acid. This two-enzyme reaction sequence is then repeated on the shortened fatty acid. Since fatty acid peroxidase attacks only fatty acids having from 13 to 18 carbon atoms, this pathway cannot lead to complete oxidation. It is believed to function as a source of long-chain fatty alcohols, which occur in large amounts in plant waxes.

The α-oxidation pathway



Summary

Free fatty acids are derived either from the bloodstream, in which they are transported bound to serum albumin, or from hydrolysis of intracellular lipids. They are first activated by esterification with CoA to form acyl CoA esters at the outer mitochondrial membrane and are then converted into O-fatty

acyl carnitine esters, which can cross the inner mitochondrial membrane into the matrix, where fatty acyl CoA esters are reformed. All subsequent steps in the oxidation of fatty acids take place upon their CoA esters within the mitochondrial matrix. The successive oxidative removal of acetyl CoA units from long-chain saturated fatty acyl CoA is called β oxidation. Four reaction steps are required to remove each acetyl CoA residue: (1) The dehydrogenation of carbon atoms 2 and 3 by FAD-linked fatty acyl CoA dehydrogenases, (2) hydration of the resulting 2,3-*trans* double bond by enoylhydratase, (3) dehydrogenation of the resulting L-β-hydroxy fatty acyl CoA by an NAD^+-linked dehydrogenase, and (4) a CoA-requiring cleavage (thiolysis) of the resulting β-keto fatty acyl CoA, to form acetyl CoA and the CoA ester of a fatty acid shortened by two carbons. The shortened fatty acid CoA ester can then reenter the sequence. For complete oxidation of the 16-carbon palmitic acid, seven cycles through the system are required, yielding eight molecules of acetyl CoA. Electrons removed in the two dehydrogenation steps flow to oxygen via the respiratory chain, accompanied by oxidative phosphorylation of ADP. The acetyl CoA formed during fatty acid oxidation is then oxidized to CO_2 and H_2O via the tricarboxylic acid cycle. The overall equation for oxidation of palmitoyl CoA is

$$\text{Palmitoyl CoA} + 23O_2 + 131P_i + 131\text{ADP} \longrightarrow 16CO_2 + 146H_2O + 131\text{ATP}$$

This process recovers about 40 percent of the standard free energy of oxidation of palmitic acid as phosphate-bond energy.

Unsaturated fatty acids require additional enzymatic steps in order to shift their double bonds into the proper position for the hydration step and to yield the L-stereoisomer of the β-hydroxy intermediate. The ketone bodies acetoacetate and β-hydroxybutyrate are formed in the liver following deacylation of acetoacetyl CoA. They are carried to other tissues, where they are oxidized via acetyl CoA and the tricarboxylic acid cycle. Odd-carbon fatty acids are oxidized to acetyl CoA and propionyl CoA. Carboxylation of propionyl CoA yields methylmalonyl CoA, which undergoes isomerization to succinyl CoA. The latter reactions require 5′-deoxyadenosylcobalamin, a coenzyme form of vitamin B_{12}, which participates in a number of enzymatic reactions as an alkyl group carrier. α and ω oxidation represent minor pathways of fatty acid catabolism.

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EFFECT OF FATTY ACIDS ON THE RESISTANCE OF MICE TO TRANSPLANTED CANCER.*

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Soon after the discovery of the fact that resistance against transplanted cancer can be produced in animals by preliminary injections of homologous living cells,[1] it was found that neither serum nor plasma has such an immunizing action, nor are killed cells potent in bringing it about.[2] These facts have lead to a general conclusion that "some subtle product of metabolism of the living cell" is essential in producing immunity, and that cell-free substances are ineffective as "antigens."[3] Nevertheless, I have been able to show recently that a state of increased resistance, in every way comparable to that induced by living cells, can be engendered in mice by the injection of a suitable quantity of olive oil.[4] This work had for its starting point the observation that a lymphoid cell reaction follows the injection of fatty oil,[5]—a reaction essentially similar to that which ensues after an injection of homologous living cells.[6,7] Pursuing the investigation, I found it

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[3] For the review of literature see Woglom, W. H., Studies in cancer and allied subjects. The study of experimental cancer, a review, New York, 1913, i.

[4] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

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possible, by a judicious use of olive oil, to induce a general stimulation of lymphoid tissue such as occurs in mice which have been rendered resistant to cancer inoculation by injections of living cells. Shortly after the appearance of my communication, Bierich[8] reported that an increased resistance to cancer transplantation could be induced by means of an unsaturated fatty acid, the name of which he failed to give.

In experiments to be reported in the present paper, I have attempted to throw a further light on the effect of fatty acids on the resistance to transplanted cancer by testing the influence of the following substances: sodium oleate, sodium palmitate, sodium stearate, oleic acid, linolic acid, and linolenic acid.

Experimental Methods.

The soaps were made up in 1 per cent solutions with distilled water and were administered in the form of a single intraperitoneal injection. Fatty acids were similarly injected in 1 per cent emulsion. In general these latter were emulsified with distilled water to which an appropriate amount of sodium hydroxide was added so as to make the emulsion fairly stable. In the case of oleic acid, the emulsion was sometimes prepared from a sodium oleate solution by adding a sufficient amount of hydrochloric acid. The oleic acid emulsion thus obtained was found not to differ in its action from the one prepared directly from oleic acid.

In testing the resistance to transplanted cancer of mice thus injected, an interval of 10 days was allowed to elapse between the injection and the implantation of cancer (Bashford Adenocarcinoma No. 63.) In each experiment, as control, a suitable number of untreated normal mice of the same breed were implanted at the same time with the same tumor. The implantation was made through a hollow needle, according to the established technique, into the subcutaneous tissue of the left groin, and the rate of growth of tumors was charted weekly thereafter. All the mice used were young adults of about the same size, weighing from 17 to 20 gm. each.

[8] Bierich, R., Leeuwenhoek-Vereeniging, 1922, I, 14.

Immunizing Action of Soaps.

My first task was to compare the action of unsaturated and saturated fatty acids. This I have attempted to do by testing sodium salts of oleic acid (unsaturated), palmitic acid, and stearic acid (saturated).

Sodium Oleate. Experiments 1 to 5.—Sodium oleate (Merck) was injected in 2 to 6 mg. amounts (0.2 to 0.6 cc. of 1 per cent solution). The incidence of resistance to the transplanted cancer exhibited by the treated mice as contrasted with that of the normal controls is shown in Table I.

TABLE I.

Effect of Sodium Oleate on Resistance to Implanted Cancer. Experiments 1 to 5.

Experiment No.	Amount of sodium oleate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.				
1	2	7	2 (28.5 per cent).	9	1 (11.1 per cent).
2	4	12	5 (41.6 " ").	9	1 (11.1 " ").
3	6	8	5 (62.5 " ").	9	2 (22.2 " ").
4	6	12	4 (33.3 " ").	10	0 (0.0 " ").
5	6	16	9 (56.2 " ").	10	1 (10.0 " ").
Average for 6 mg.....		36	18 (50.0 per cent).	29.	3 (10.3 per cent).

The above results demonstrate that injections of sodium oleate in amounts of 4 to 6 mg. each produce a material increase in the resistance of mice to subsequent cancer transplantation. All the mice were in good physical condition, though an amount of sodium oleate larger than that used in these experiments, if given in a single injection, produced ill effects on their general health.

Sodium Palmitate. Experiments 6 to 11.—Sodium palmitate (Merck) was injected into mice in the form of 1 per cent solution in amounts varying from 2 to 12 mg. Cancer implants were made 10 days after the soap injection. The results of six experiments were as follows (Table II):

TABLE II.

Effect of Sodium Palmitate on Resistance to Implanted Cancer. Experiments 6 to 11.

Experiment No.	Amount of sodium palmitate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.				
6	2	8	0 (0.0 per cent).	9	1 (11.1 per cent).
7	4	10	2 (20.0 " ").	9	1 (11.1 " ").
8	6	10	3 (30.0 " ").	9	2 (22.2 " ").
9	8	10	2 (20.0 " ").	9	2 (22.2 " ").
10	10	9	1 (11.1 " ").	8	2 (25.0 " ").
11	12	10	2 (20.0 " ").	8	2 (25.0 " ").
Average.....		57	10 (17.5 per cent).	52	10 (19.2 per cent).

It is evident that sodium palmitate, unlike sodium oleate, does not exert an immunizing action on mice against transplanted cancer, at least when given in the amounts employed in the above experiments.

Sodium Stearate. Experiments 12 to 17.—Various amounts of sodium stearate (Merck) were injected into mice in the form of 1 per cent solution and the resistance of the animals subsequently tested. Table III gives the results.

TABLE III.

Effect of Sodium Stearate on Resistance to Implanted Cancer. Experiments 12 to 17.

Experiment No.	Amount of sodium stearate.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	mg.				
12	2	10	2 (20.0 per cent).	7	1 (14.2 per cent).
13	4	7	1 (14.2 " ").	7	1 (14.2 " ").
14	6	10	2 (20.0 " ").	11	2 (18.1 " ").
15	8	10	1 (10.0 " ").	11	2 (18.1 " ").
16	10	8	2 (25.0 " ").	9	2 (22.2 " ").
17	12	6	1 (16.6 " ").	9	2 (22.2 " ").
Average.....		51	9 (17.6 per cent).	54	10 (18.5 per cent).

As in the case of sodium palmitate, no perceptible difference can be noted in the incidence of resistance in mice treated with sodium stearate and that of untreated controls.

Immunizing Action of Unsaturated Fatty Acids.

The foregoing experiments show that sodium oleate has a marked immunizing action on mice against transplanted cancer, and that sodium palmitate and sodium stearate are wholly without such effect. These observations, coupled with our previous results with olive oil[4] lead one to question whether oleic acid may not be responsible for the positive findings. In order to test this point and to determine, furthermore, the influence of unsaturation in the reaction, we have tested three unsaturated fatty acids, representatives of three different chemical series; *viz*, oleic acid, linolic acid, and linolenic acid.

Oleic Acid. Experiments 18 to 23.—A 1 per cent emulsion of oleic acid was injected intraperitoneally into mice in doses varying from 0.4 to 0.8 cc. each (0.004 to 0.008 cc. of oleic acid). In Experiments 18, 20, and 23, the emulsion used was prepared from sodium oleate (Merck) in the manner already described. The emulsion used for other experiments was made directly from oleic acid (Eimer and Amend). The cancer implantation was carried out 10 days after the injection of the emulsion. The results are summarized in Table IV.

TABLE IV.

Effect of Oleic Acid on Resistance to Implanted Cancer. Experiments 18 to 23.

Experiment No.	Amount of oleic acid.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	cc.				
18	0.004	9	3 (33.3 per cent).	6	1 (16.6 per cent).
19	0.004	10	8 (80.0 " ").	10	2 (20.0 " ").
20	0.005	10	7 (70.0 " ").	12	2 (16.6 " ").
21	0.006	8	6 (75.0 " ").	6	1 (16.6 " ").
22	0.006	11	5 (45.4 " ").	10	0 (0.0 " ").
23	0.008	13	8 (61.5 " ").	11	4 (36.3 " ").
Average.....		61	37 (60.6 per cent).	55	10 (18.1 per cent).

The results of these experiments provide conclusive evidence that oleic acid is highly potent in increasing the resistance of mice to transplanted cancer. It is noteworthy that this action of oleic acid was obtained with all of the various amounts employed.

Linolic Acid. Experiments 24 to 30.—A 1 per cent linolic acid (Kahlbaum) emulsion was injected into mice in amounts varying from 0.2 to 0.8 cc. each (0.002 to 0.005 cc. of linolic acid). The resistance to transplanted cancer shown by the treated mice as contrasted to that of untreated controls is recorded in Table V.

TABLE V.

Effect of Linolic Acid on Resistance to Implanted Cancer. Experiments 24 to 30.

Experiment No.	Amount of linolic acid.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	cc.				
24	0.002	10	3 (30.0 per cent).	10	4 (40.0 per cent).
25	0.004	10	5 (50.0 per cent).	10	2 (20.0 per cent).
26	0.004	10	5 (50.0 " ").	10	2 (20.0 " ").
27	0.005	10	6 (60.0 " ").	12	2 (16.6 " ").
28	0.006	10	6 (60.0 " ").	10	2 (20.0 " ").
29	0.006	10	5 (50.0 " ").	10	2 (20.0 " ").
Average for 0.004 to 0.005 cc.		30	27 (51.0 per cent).	52	10 (19.2 per cent).
30	0.008	7	3 (42.8 per cent).	11	3 (27.2 per cent).

It is evident from the above figures that linolic acid is capable of producing increased resistance to cancer inoculation, and that the optimum doses are from 0.004 to 0.005 cc. each.

Linolenic Acid. Experiments 31 to 37.—Linolenic acid (Kahlbaum) was injected into mice in the form of a 1 per cent emulsion in varying amounts. The outcome of cancer implantation 10 days after the linolenic acid injection was as follows (Table VI):

TABLE VI.

Effect of Linolenic Acid on Resistance to Implanted Cancer. Experiments 31 to 37.

Experiment No.	Amount of linolenic acid.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	cc.				
31	0.002	10	5 (50.0 per cent).	10	4 (40.0 per cent).
32	0.004	10	10 (100.0 per cent).	10	4 (40.0 per cent).
33	0.004	10	6 (60.0 " ").	10	2 (20.0 " ").
34	0.005	10	4 (40.0 " ").	12	2 (16.6 " ").
Average for 0.004 to 0.005 cc.		30	20 (66.6 per cent).	32	8 (25.0 per cent).
35	0.006	10	2 (20.0 per cent).	11	4 (36.3 per cent).
36	0.006	6	1 (16.6 " ").	10	2 (20.0 " ").
37	0.008	9	3 (33.3 " ").	11	4 (36.3 " ").

The above result warrants the conclusion that linolenic acid shares, with other unsaturated fatty acids already tested, the immunizing action against subsequent cancer inoculation. The range of optimum doses here is apparently somewhat less than in the case of linolic acid and it is decidedly less than that of oleic acid.

Influence upon Previously Implanted Cancer Grafts.

With the immunizing action of unsaturated fatty acids against subsequent cancer inoculation established, attention was next turned to the possible effect of these substances on the growth of cancer grafts already implanted.

Sodium Oleate. Experiments 38 to 41.—Mice were implanted with Bashford adenocarcinoma in the usual manner. They were divided into two groups, and the mice in one group were injected with 0.5 cc. of 1 per cent solution of sodium oleate 2 or 48 hours after the implantations. The other group remained untreated and served as control. The results of four similar experiments are summarized in Table VII.

TABLE VII.

Effect of Sodium Oleate on Cancer Grafts Previously Implanted. Experiments 38 to 41.

Experiment No.	Interval between tumor inoculation and the soap injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
38	2	15	7 (46.6 per cent).	10	3 (30.0 per cent).
39	2	7	3 (42.8 " ").	10	2 (20.0 " ").
40	48	20	3 (15.0 " ").	10	0 (0.0 " ").
41	48	8	1 (12.5 " ").	8	0 (0.0 " ").
Average.....		50	14 (28.0 per cent).	38	5 (13.1 per cent).

Oleic Acid. Experiments 42 to 44.—Similar experiments were carried out to test the effect of oleic acid, injected in 0.5 cc. of 1 per cent emulsion following the cancer implantation. Table VIII summarizes the result.

TABLE VIII.

Effect of Oleic Acid on Cancer Grafts Previously Implanted. Experiments 42 to 44.

Experiment No.	Interval between tumor inoculation and oleic acid injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
42	2	15	6 (40.0 per cent).	10	3 (30.0 per cent).
43	48	7	2 (28.5 " ").	7	1 (14.2 " ").
44	48	9	4 (44.4 " ").	10	2 (20.0 " ").
Average.....		31	12 (38.7 per cent).	27	6 (22.2 per cent).

Linolic Acid. Experiments 45 to 47.—The effect of 0.5 cc. of 1 per cent emulsion of linolic acid was similarly tested in three experiments (Table IX).

TABLE IX.

Effect of Linolic Acid on Cancer Grafts Previously Implanted. Experiments 45 to 47.

Experiment No.	Interval between tumor inoculation and linolic acid injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
45	2	10	3 (30.0 per cent).	10	2 (20.0 per cent).
46	48	9	3 (33.3 " ").	7	1 (14.2 " ").
47	48	18	4 (22.2 " ").	10	1 (10.0 " ").
Average.....		37	10 (27.0 per cent).	27	4 (14.8 per cent).

Linolenic Acid. Experiments 48 to 50.—In these experiments the effect of 0.5 cc. of 1 per cent emulsion of linolenic acid was tested as in the preceding experiments (Table X).

TABLE X.

Effect of Linolenic Acid on Cancer Grafts Previously Implanted. Experiments 48 to 50.

Experiment No.	Interval between tumor inoculation and linolenic acid injection.	Treated mice.		Controls.	
		Number.	Resistant mice.	Number.	Resistant mice.
	hrs.				
48	2	10	2 (20.0 per cent).	10	2 (20.0 per cent).
49	48	8	3 (37.5 " ").	7	1 (14.2 " ").
50	48	20	2 (10.0 " ").	10	1 (10.0 " ").
Average.....		38	7 (18.4 per cent).	27	4 (14.8 per cent).

It is evident from the above results that no marked effect can be produced by any of these means on cancer grafts already implanted. There was a slightly lower per cent of tumor development in the treated mice than in the controls, but the difference by itself was too small to be considered significant. However, in treated mice, there were always two or three out of every eight or nine tumors that grew extremely slowly, or almost not at all. Upon microscopical examination these tumors were found to consist of small, discrete masses of

neoplastic cells surrounded by a dense layer of connective tissue with intense lymphocytic infiltration. No such retardation of growth occurred in tumors of the control series, all growing progressively. These findings suggest, rather than prove, that the fatty acids do exert some influence but that this is not adequate to modify markedly the development of a rapidly growing cancer such as the one used in these experiments. It may be recalled that all the hitherto discovered methods of inducing general resistance against subsequent cancer grafting fail when tested against grafts already *in situ*. This is probably because of the great rapidity with which most of the transplantable cancers grow, as also because the artificially induced state of resistance develops only after a latent period of several days.[1,4,9]

DISCUSSION.

The experiments reported in the present paper show conclusively that unsaturated fatty acids such as oleic, linolic, and linolenic acids have a distinct immunizing action against transplanted cancer in mice, and that this action is apparently not shared by saturated fatty acids. Of the unsaturated fatty acids used, oleic acid shows the widest range in the amounts capable of producing the desired effect, while the range with the other acids is more limited. There is no apparent difference in the activity of these unsaturated fatty acids in the optimum amounts. Whether the immunity-producing quality of fats can be estimated on the basis of their iodine values remains to be determined. In an unpublished observation it was noted that cocoanut oil which has an extremely low iodine value produces no resistance to cancer, and at the same time that cod liver oil, in spite of a very high iodine value, is equally impotent in this regard. However, it must be remembered that in dealing with such complex mixtures as these fats, factors other than fatty acids must be taken into consideration in interpreting biological effects.

A problem of broad interest suggested by these results is that of the possible part played by unsaturated fatty acids in cancer immunity induced by means other than injection of them. In addition to the

[9] Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

older method of living tissue injection,[1] it has been possible to induce immunity by exposing mice to suitable doses of x-radiation[10, 11] or to intense dry heat.[12] It seems not unreasonable to suspect that these varied treatments may be at one in producing disturbances that involve some changes in the unsaturated fatty acid.

The possibility that the cancer immunity induced by unsaturated fatty acids may be consequent on increased activity of the lymphoid tissue deserves consideration. The close relationship existing between fat metabolism and the lymphoid tissue,[13] under both physiological and pathological conditions, and the clearly demonstrated association of lymphoid stimulation with cancer immunity[7, 11, 12, 14] are in favor of this view. As a matter of fact, we have found that injections of sodium oleate in amounts sufficient to produce immunity bring about a marked increase in the number of karyokinetic figures in the lymphoid tissue, indicating an increased proliferative activity of this tissue.

SUMMARY.

Sodium oleate, oleic acid, linolic acid, and linolenic acid injected into mice in suitable amounts induce a material increase in the resistance against subsequent transplantation of cancer grafts, although they fail to exert so marked an influence on cancer grafts already in place.

Sodium palmitate and sodium stearate, on the other hand, do not produce immunity, at least in the amounts employed in the present study.

[10] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

[11] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[12] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25.

[13] Schäfer, E. A., *Internat. Monatschr. Anat. u. Histol.*, 1885, ii, 6. Bergel, S., *Ergebn. inn. Med. u. Kinderheilk.*, 1921, xx, 36.

[14] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

EFFECT OF SODIUM OLEATE ON THE COURSE OF EXPERIMENTAL ATHEROSCLEROSIS IN RABBITS

N. A. Novikova

In an earlier work, we observed a rather rapid reverse development of alimentary hypercholesterolemia and decrease in deposition of lipids in the aortic wall of rabbits with experimental atherosclerosis after they received sodium oleate.* Because the literature on the subject is contradictory, we undertook this work to study the effect of sodium oleate on the development and course of experimental atherosclerosis in rabbits injected with cholesterol at the same time.

Experiments were performed on 22 male rabbits weighing 2.5 to 3 kg. All the animals received every other day for 4 months 1 g of cholesterol in the form of a 20% solution in sunflower oil (5 ml of the solution) along with root vegetables. All the animals were divided into 2 groups a month after the start of feeding. The first group consisted of 12 rabbits. Along with cholesterol they received 20 ml of a 2% sodium oleate solution 3 times a week (1.2 g each time) for 3 months. The 10 rabbits of the second group continued to receive only cholesterol (the control).

*N. A. Novikova, Yezhegodnik IEM AMN SSSR za 1961-1963 (Yearbook of the Institute of Experimental Medicine, USSR Academy of Medical Sciences, for 1961-1962), Leningrad, 1963, Vol. 7-8, pt. 2, p. 226.

The levels of total serum cholesterol and phospholipids were investigated every month in all the animals, which were killed with an aeroembolism 4 months from the time they first received cholesterol. Total lipids were determined in the aortas by the method of Buck and Rossiter* as modified by N. G. Stroykova, L. V. Ivanova, and G. P. Fedorova*, while the cholesterol content was determined from the Liebermann Burchardt color reaction. Liver cholesterol was investigated by Bloor's test. The resulting data were statistically analyzed.

One month after the start of feeding of cholesterol, no differences were noted between the 2 groups of rabbits as far as the rise in blood cholesterol and pholipids was concerned. After sodium oleate was administered simultaneously with cholesterol, the cholesterol content tended to increase more rapidly. It is evident from Fig. 1 that blood cholesterol in the control averaged 541±79 mg% after 4 months compared to 875±93 mg% ($P < 0.02$) in the experimental rabbits. Serum phospholipids in the experimental animals was 208±25 mg% after months and differed little from the control, 183±18 mg% ($P > 0.2$). Examination of the aortas showed that they weighed somewhat more on average in the experimental rabbits (626±54 mg) than in the control (587±52 mg). Gross inspection of the atherosclerotic lesions in t aortas revealed the greatest changes in the animals that received sodium oleate in addition to cholesterol. Biochemical analysis s

*Buck, R. and R. Rossiter, Arch. Path., 1954, Vol. 51, No. 3, P.
**Stroykova, L. V. Ivanova, and G. B. Fedorova, Yezhegodnik IEM Leningrad, 1960, P. 444.

that these animals had a very high lipid content in the aortic walls (3.9±0.8 mg)* while the control had almost half as much, 2±0.3 mg, $P > 0.05$ (Fig. 2, I). When the cholesterol fractions were isolated from the total lipids, it was found that the cholesterol levels in the experimental animals was more than 3 times as high on the average (1.87±0.47 mg) as in the control, 0.55±0.11 mg, $P > 0.02$ (Fig. 2, II). The cholesterol level of liver tissue was much higher in the experimental animals (2069±237 mg%) than in the control, 1454±276 mg%, $P > 0.001$, Fig. 2, III)

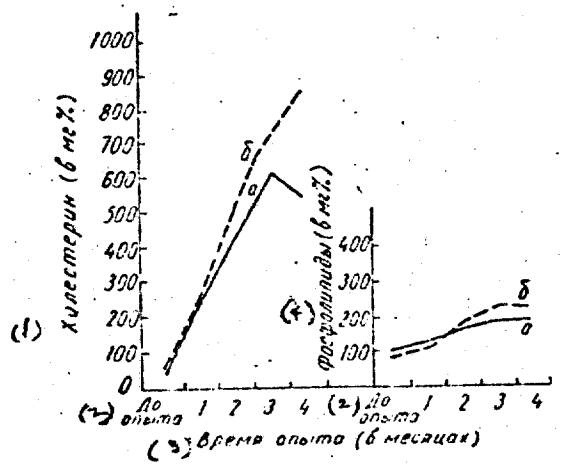

Fig. 1. Effect of sodium oleate on rabbit serum cholesterol and phospholipids in experimental atherosclerosis.

a - control group; b - experimental group

1 - Cholesterol (in mg%)

2 - Before the experiment

3 - Time of the experiment (in months)

4 - Phospholipids (in mg%)

*The content of total lipids and cholesterol in the aortic wall is expressed in milligrams per 100 mg of wet aortic tissue.

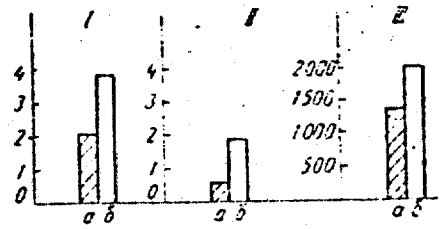

Fig. 2. Average content (in mg%) of total lipids (I) and cholesterol (II) in the aortic wall and cholesterol level of liver tissue (III).

a - control group; b - experimental group

Thus, our data show that sodium oleate promotes a fairly rapid increase in alimentary hypercholesterolemia and intensifies the depositio of lipids and cholesterol in the aorta and liver of rabbits.

Several investigators who studied the effect of various fatty acids on the absorption of cholesterol in the intestine and its excretion from the body show that oleic acid accelerates the absorption of exogenou cholesterol in the intestine while slowing the excretion of endogenous cholesterol. The intensification of experimental atherosclerosis in our experimental rabbits given sodium oleate simultaneously with cholesterol may have been caused by the accelerated absorption of cholesterol and slowing of its excretion due to the oleic acid and by the increased deposition of cholesterol esters with oleic acid in plaques, as some investigators have observed.

Vopr. Pitan., 27(4): 82-83 (1968)

УДК 616.13-004.6-092.9-085.31:547.1924.1

ВЛИЯНИЕ НАТРИЕВОЙ СОЛИ ОЛЕИНОВОЙ КИСЛОТЫ НА ТЕЧЕНИЕ ЭКСПЕРИМЕНТАЛЬНОГО АТЕРОСКЛЕРОЗА У КРОЛИКОВ

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В предыдущей работе мы обнаружили более быстрое обратное развитие алиментарной гиперхолестеринемии и уменьшение отложений липидов в стенке аорт при введении натриевой соли олеиновой кислоты кроликам с экспериментальным атеросклерозом[2]. В связи с тем что литературные сведения по данному вопросу противоречивы, мы провели настоящую работу с целью изучить влияние натриевой соли олеиновой кислоты на развитие и течение экспериментального атеросклероза у кроликов в условиях введения ее одновременно с холестерином.

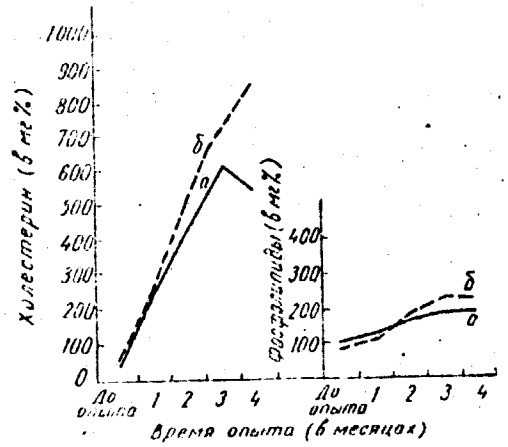

Рис. 1. Влияние натриевой соли олеиновой кислоты на содержание холестерина и фосфолипидов в сыворотке крови кроликов при экспериментальном атеросклерозе.

а — контрольная группа; *б* — опытная группа.

Опыты проводили на 22 кроликах-самцах весом 2,5—3 кг. Все кролики через день в течение 4 месяцев получали с корнеплодами по 1 г холестерина в виде 20% раствора в подсолнечном масле (5 мл раствора). Через месяц от начала кормления холестерином всех кроликов разделили на 2 группы.

В 1-й группе было 12 кроликов. Одновременно с холестерином они 3 раза в неделю получали по 20 мл 2% раствора натриевой соли олеиновой кислоты (1,2 г в неделю) в течение 3 месяцев. Кролики 2-й группы (10) продолжали получать только холестерин (контроль).

Ежемесячно у всех животных исследовали содержание общего холестерина и фосфолипидов в сыворотке крови. Через 4 месяца от начала кормления холестерином всех кроликов забивали воздушной эмболией. В аортах определяли содержание общих липидов по методу Бляйха и Росситера[1] в модификации Н. Г. Стройковой, Л. В. Ивановой и Г. Н. Федоровой[2] и содержание холестерина по цветной реакции Либермана-Бурхарда. Содержание холестерина в печени исследовали методом Блюра. Полученные данные подвергнуты статистической обработке.

Через месяц после начала кормления холестерином у кроликов 1-й и 2-й группы не отмечено различий в повышении уровня холестерина и фосфолипидов в сыворотке крови. После введения одновременно с холестерином натриевой соли олеиновой кислоты появилась тенденция к более быстрому увеличению содержания холестерина. Как видно из рис. 1, содержание холестерина в сыворотке крови у животных контрольной группы через 4 месяца составляло в среднем 541±79 мг%, а у кроликов опытной группы — 875±93 мг% ($P<0,02$). Содержание фосфолипидов в сыворотке крови подопытных животных через 4 месяца равнялось 208±25 мг% и существенно не отличалось от такового кроликов контрольной группы — 183±18 мг% ($P>0,2$). При исследовании аорт обнаружено, что вес их у подопытных кроликов в среднем несколько больше (626±54 мг), чем у контрольных (587±52 мг). При визуальной оценке степени атеросклеротических поражений в аортах наибольшие изменения выявлены у животных, получавших дополнительно к холестерину натриевую соль олеиновой кислоты.

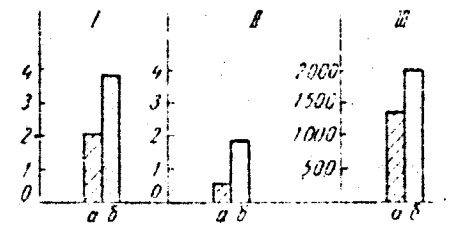

Рис. 2. Среднее содержание (в мг%) общих липидов (*I*) и холестерина (*II*) в стенке аорты, а также холестерина в ткани печени (*III*).

а — контрольная группа; *б* — опытная группа.

Данные биохимического исследования показали, что у животных этой группы было очень высокое содержание липидов в стенке аорт (3,9±0,8 мг)[3], у контрольных кроликов оно оказалось почти в 2 раза меньше — 2±0,3 мг; $P>0,05$ (рис. 2, *I*). При выделении фракции холестерина из общих липидов аорт установлено, что содержание холестерина у подопытных животных в среднем было более чем в 3 раза выше (1,87±0,47 мг), чем у контрольных — 0,55±0,11 мг; $P>0,02$ (рис. 2, *II*). У кроликов опытной группы уровень холестерина в ткани печени оказался значительно выше (2069±237 мг%), чем у контрольных — 1454±276 мг%; $P>0,001$ рис. 2, *III*).

Таким образом, полученные данные свидетельствуют, что натриевая соль олеиновой кислоты способствует более быстрому нарастанию пищевой гиперхолестеринемии и усилению отложений липидов и холестерина в аорте и печени кроликов.

Некоторые исследователи изучали влияние различных жирных кислот на всасывание холестерина в кишечнике и его выделение из организма и показали, что олеиновая кислота вызывает ускорение всасывания, экзогенного холестерина в кишечнике и задерживает выделение эндогенного холестерина из организма. Возможно, усиление развития экспериментального атеросклероза у кроликов и в наших опытах, в условиях одновременного введения натриевой соли олеиновой кислоты с холестерином, связано с ускорением всасывания холестерина, задержкой его выведения из организма под влиянием олеиновой кислоты, а также с увеличением отложений эфиров холестерина с олеиновой кислотой в бляшки, что отмечает ряд авторов.

Поступила 3/VII 1967 г.

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[1] S. Moore, D. H. Spackman, W. H. Stein, Anal. Chem., 1958, v. 30, N 7, c. 1185.

[2] Н. А. Новикова. Ежегодник ИЭМ АМН СССР за 1961—1962 гг. Л., 1963, т. VII—VIII, ч. 2, с. 226.

P 83

Acta pharmacol. et toxicol. 1961, 18, 141–152.

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Pharmacological Effects of Fatty Acids, Triolein and Cottonseed Oil.

By

Lars Orö and Arvid Wretlind

(Received March 8, 1961

Various triglycerides in emulsion show marked pharmacological properties on intravenous administration. For example, several with low molecular weights have been found distinctly toxic on intravenous injection (WRETLIND 1957 b; ORÖ, WESTERHOLM & WRETLIND 1959). On the other hand, fats of higher molecular weights, such as cottonseed oil, triolein and certain vegetable fats, show little or no toxicity on intravenous injection into mice. Hence it is possible to produce injectable emulsions of cottonseed oil as a carrier of agents, such as higher fatty acids, that owing to insolubility could not otherwise be studied for their pharmacological effects. Such fatty acid emulsions have enabled us to determine the toxicity of fatty acids on intravenous injection.

In the same investigation we have also studied the effects of these emulsions on respiration and blood pressure in cats. It was found that emulsions containing only cottonseed oil or triolein could cause reduced blood-pressure and apnoea. These effects were characterised by such pronounced tachyphylaxis that frequently they were noticeable only on the first injection. They were readily distinguishable, therefore, from the affects of the different fatty acid emulsions in which cottonseed oil acted as carrier.

Lastly, we conducted experiments on guinea pig small intestine in order to study the effects of fatty acid emulsions on smooth muscle.

Experimental.

Animal Experiments.

For *determinations of toxicity*, mice weighing 13–34 g were used. The emulsions or solutions of fatty acids were injected into the tail vein. Each fatty acid was administered to at least six groups each of 10 animals. The calculations of LD50 with their standard

errors were by the method of MILLER & TAINTER (1944); the results are summarised in figure 1.

Some 100 cats weighing 2–6 kg were used for investigating the *respiratory* and *circulatory* responses to cottonseed oil, triolein and fatty acid emulsions. These animals were anaesthetized with diallymal (allobarbital (WHO), dial ®, Ciba, 25–40 mg per kg body weight) or chloralose (50 mg/kg). Respiration and blood pressure were recorded by means of a Grass electroencephalograph[1]) with supplementary demodulators. A Statham pressure transducer, model P 23 AA[2]), was used for blood-pressure determinations on the carotid artery. The respiration was recorded with a Grass pressure transducer PT-5 connected to a side tube in a tracheal cannula.

In a number of experiments the *pulmonary arterial pressure* was measured via a plastic catheter that had been sutured in the artery after opening the thorax. In one experiment the thorax was closed again; in the others it was left open under artificial respiration.

For studying the direct effect of fatty acids upon the heart, a *heart-lung preparation* described by WRETLIND (1957 b) was used. Blood pressure and flow were recorded by the Grass transducers mentioned above.

The action of fatty acids on guinea pig *small intestine* was studied in an isolated organ bath containing 15 ml Tyrode solution (0.8 % NaCl; 0.02% KCl; 0.02% $CaCl_2$; 0.02% $MgCl_2 \cdot 6\,H_2O$; 0.1% $NaHCO_3$; 0.005% $NaH_2PO_4 \cdot H_2O$; and 0.1% glucose). The organ bath was aerated with air. A Grass force-displacement transducer, model FT-03, was employed for recording the tension of the gut. In this connection we also studied the ability of fatty acids to abolish contractions elicited by histamine. For this purpose histamine (0.2 µg/15 ml) was first added to the organ bath and fatty acid emulsion was added without prior washing when maximal contraction had been obtained. The observed relaxation of the intestine was recorded.

Test Solutions and Test Emulsions.

For the *toxicity determinations* in mice the lower *fatty acids* (acetic, propionic, butyric, isovaleric, norvaleric, caproic and heptylic) were administered in aqueous solution, whereas the higher fatty acids, because of their insolubility in water, were emulsified. To prepare these *emulsions* the fatty acids were first dissolved in heated cottonseed oil, which served as carrier. Phosphatides, sodium cholate and glycerin monostearate were used as emulsifiers (table 1). The emulsions were homogenized in a Logeman apparatus (WRETLIND 1957 a). The pH of the solutions and emulsions was adjusted to 7.3 with NaOH.

The concentrations of fatty acids were so chosen that the injected volume required for determining LD50 amounted to between 3 and 45 ml per kg body weight. Thus acetic, propionic, butyric, isovaleric, norvaleric and caproic acids were administered as 10% and heptylic acid as 5% aqueous solutions; caprylic, nonanoic, capric, undecylic, lauric, tridecylic and myristic acids as 2% emulsions; pentadecylic, palmitic, margaric and stearic acids as 0.2%, and oleic acid as 2% emulsions.

For investigating the action of fatty acids on *guinea-pig gut*, 1% emulsions of the compositions recorded in table 1 were used.

Cottonseed oil was similarly utilized as carrier when investigating the *respiratory* and *blood-pressure* responses of the cat to higher fatty acids. Emulsification was with phosphatides and Pluronic F 68 (table 2), because they make stabler emulsions of

1) Grass Instrument Co., Quincy, Mass., U.S.A.
2) Statham Instruments Inc., 254 Carpenter Road, Hato Rey, Puerto Rico.

Table 1.

Composition of fatty acid emulsions used for determining the toxicity in mice.

The cottonseed oil, supplied by Wesson Oil and Snowdrift Sales Co., New Orleans, La., U.S.A., was used after filtration at +4°C. The phosphatides were prepared as described WRETLIND (1957 a). The emulsions were adjusted to pH 7.3 with N-NaOH.

Fatty acid	0.2–2 g
Cottonseed oil	10 g
Phosphatides	0.4 g
Sodium cholate	0.1 g
Glycerol monostearate	0.5 g
5% glucose solution to	100 ml

higher fatty acids than the emulsifiers previously mentioned. The fatty acids investigated were stearic, oleic, lauric and tridecylic.

The *cottonseed oil emulsions* used in the cat experiments had the compositon shown in table 2, except that they contained no fatty acid and that either unsaturated phosphatides (WRETLIND 1957 a) or hydrogenated phosphatides were used. In some experiments we studied emulsions containing triolein instead of cottonseed oil.

The *hydrogenated phosphatides* were prepared as described below. The phosphatides 320 g were dissolved in 600 ml cyclohexane: 3,400 ml absolute alcohol were added, and the precipitated cephalins were filtered off. The filtrate was concentrated under reduced pressure and nitrogen to a volume of 850 ml. To the resulting solution were added 30 mg Raney nickel as catalyst, the solution being then transferred to an autoclave in which it was subjected to hydrogenation at 85–130 C with hydrogen at a pressure of 75–150 atmospheres for two hours. The hydrogenated solution was filtered hot and passed through a column of 500 g Al_2O_3 with a diameter of 45 mm, equipped with a heating jacket (75°). The column was eluted with 95% ethanol. The first fraction of 300 ml was discarded; the next two litres of eluate were added to six litres off acetone and placed in a refrigerator at +4°. The resulting phosphatides were filtered of under reduced pressure, washed with acetone, and dried in a desiccator under reduced pressure. The yield amounted to 30–50 grams. Analysis showed N 1.6–1.7 per cent and P 3.7–3.8 per cent.

Table 2.

Composition of emulsions used for investigating the effects of fatty acids on respiration and blood pressure.

Pluronic F 68 (polypropylene-polyethylene-glucose) obtained from Wyandotte Chemical Corp., Wyandotte, Ill., U.S.A. The emulsions were adjusted to pH 7.3 with 1 N-NaOH.

Fatty acid	1 g
Cottonseed oil	10 g
Phosphatides	1.2 g
Pluronic	0.4 g
5% glucose solution to	100 ml

Results.

In attempts to determine the LD50 for the carrier emulsions with *cottonseed oil alone*, we found on intravenous injection of a maximal volume – i.e., 60 ml/kg of the 25% cottonseed oil emulsion with emulsifiers, as shown in table 1 – *into mice* only a transient depression of the respiration rate. It follows that the LD50 is higher than 15 g cottonseed oil per kilogram.

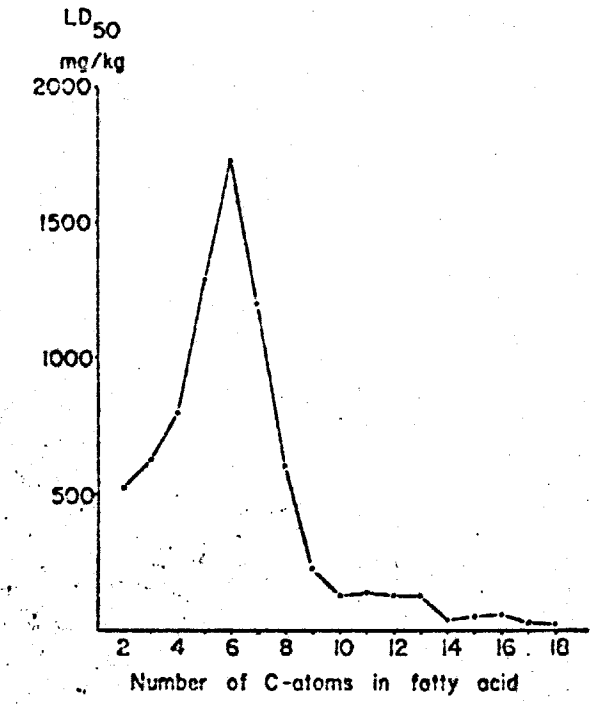

Fig. 1. *Toxicity of saturated fatty acids on intravenous injection into mice.* The abscissa indicates the number of carbon atoms in the fatty acid molecule; the ordinate, LD50 in milligrams per kilogram of body weight. The LD50 values in mg/kg, together with their standard errors, were: acetic acid 525 ± 21; propionic acid 625 ± 33; butyric acid 800 ± 24; n-valeric acid 1290 ± 53; isovaleric acid 1120 ± 30; caproic acid 1725 ± 85; heptylic acid 1200 ± 56; caprylic acid 600 ± 24; nonanoic acid 224 ± 4.6; capric acid 129 ± 5.4; undecylic acid 140 ± 4.2; lauric acid 131 ± 5.7; tridecylic acid 130 ± 7.0; myristic acid 43 ± 2.6; pentadecylic acid 54 ± 3.2; palmitic acid 57 ± 3.4; margaric acid 36 ± 0.3; stearic acid 23 ± 1.3.

Figure 1 shows that the intravenous *toxicities of different fatty acids* differ considerably for mice. Least toxic is caproic acid with six carbon atoms, which has an LD50 of 1,725 ± 85 mg per kg body weight. With an increased or decreased number of carbon atoms in the fatty acid molecule, the toxicity rises. The most toxic of all those investigated was

stearic acid, which had an LD50 of 23 ± 0.7 mg per kg. Approximately the same value was obtained for stearic acid in the rat (21.5 ± 1.8 mg/kg). Oleic acid showed an LD50 of 230 ± 18 mg/kg in mice. This implies that stearic acid is ten times more toxic than the unsaturated oleic acid. The toxicity of norvaleric and isovaleric acids differed slightly, LD50 being 1,290 ± 53 and 1,120 ± 30 mg/kg, respectively.

When the fatty acid solutions and emulsions were injected in amounts near the LD50 dose, the animals immediately had convulsions and collapsed on their sides. Respiration ceased within 1–2 minutes, sometimes after hyperpnoea.

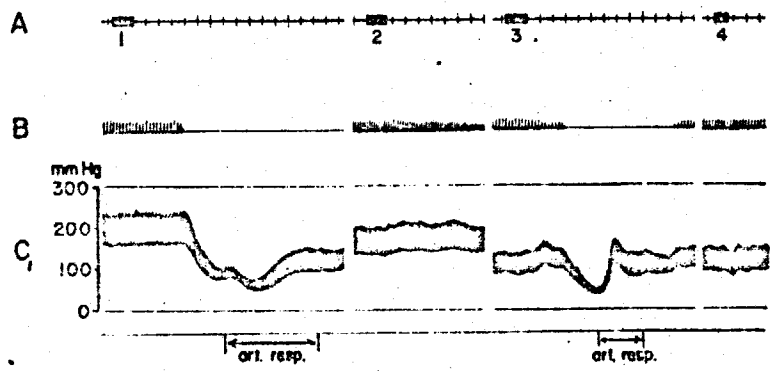

Fig. 2. *Influence of cottonseed oil emulsion on blood pressure and respiration.* Cat, 3.0 kg, under Dial ⊕ anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. "Art. resp." denotes artificial respiration. At 1, 2, 3 and 4, 0.7 ml/kg of 10% cottonseed oil emulsion containing phosphatides was injected intravenously. The interval between 1 and 2 was four minutes, between 2 and 3 four hours and between 3 and 4 two minutes.

Intravenous administration of emulsion with *cottonseed oil or triolein alone to anaesthetized cats* produced various effects, depending on the dose and the emulsifiers. With soy-bean phosphatides and Pluronic F 68 as emulsifiers an initial intravenous injection (0.2–1 ml/kg body weight) sometimes caused a fall of blood pressure as well as apnoea (fig. 2). The latency was usually around 10–40 seconds. Another injection of the same dose invariably had either a less effect or none at all. – The action of these cottonseed oil emulsions is thus characterized by pronounced tachyphylaxis; hence the same or progressively higher doses can be repeated with no demonstrable effects on blood pressure and respiration. – When Pluronic F 68 alone was the emulsifier, 1–3 ml/kg was required to produce an effect with 10% cottonseed oil or triolein emulsion.

Cottonseed oil emulsions produced a rise of pressure in the *pulmonary artery* up to 100 mm Hg (fig. 3). The latency varied from 10 seconds to

the cottonseed oil, but on repeated injection these quickly subsided because of the tachyphylaxis. With fatty acid emulsions containing hydrogenated phosphatides, only the effects of the fatty acids were observed.

Intravenous injection of the *fatty acids* investigated – stearic, oleic, lauric and tridecylic – had a hypotensive effect on the systemic *circulation*, but raised the pulmonary arterial pressure. The doses required for this action were 5 mg stearic acid, 50 mg oleic acid, 75 mg lauric acid or 60 mg tridecylic acid per kg body weight. All of the fatty acids studied had a negatively inotropic effect on the heart, as shown by the experiments on heart-lung preparations. Similar results were given by oleic acid emulsions

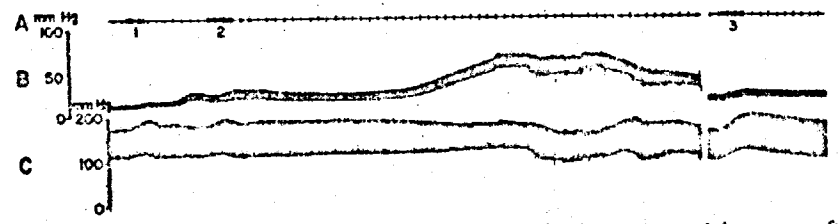

Fig. 5. *Influence of triolein emulsion on blood pressure and pulmonary arterial pressure after vagotomy.* Cat, 4.0 kg, under Dial ⊕ anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, the pressure in the pulmonary artery; C, the arterial blood pressure in mm Hg. Four ml/kg of 10% triolein emulsion with pluronic F 68 only as emulsifier were injected intravenously at 1, and 7 ml/kg at 2 and 3. It will be seen that the emulsion caused a rise of blood pressure in the pulmonary artery with a slight secondary fall in blood pressure, effects that disappeared on repeated injection.

without cottonseed oil as carrier and also by those with unsaturated phosphatides and Pluronic F 68 as emulsifiers. Emulsions with different emulsifiers differed somewhat in their effects. For instance, the effect on pulmonary arterial pressure was far less with hydrogenated than with unsaturated phosphatides. The action on the *respiration* was characterized by hyperpnoea after small doses and apnoea after larger ones. No tachyphylaxis was observed with fatty acid emulsions. In several experiments, indeed, an increased effect was noted on repeated injection of equal doses (figures 6 and 7). – Vagotomy had no effect on falls in blood-pressure produced by the fatty acids studied.

As to the action of *fatty acid emulsions on small intestine from the guinea pig*, our results indicated that the emulsions did not themselves cause any contraction, even in relatively large doses (100 mg/15 ml). They could, however, inhibit the contraction produced by histamine. The degree of inhibition depended on the particular fatty acid contained in the emulsion. Fig. 8 shows the relative potencies of the fatty acids in this respect. It will be seen from the curve that capric and undecylic acids, i.e., those with 10 or 11 carbon atoms, were the most effective. Somewhat more than 15 mg/15 ml of these acids was required for total inhibition of

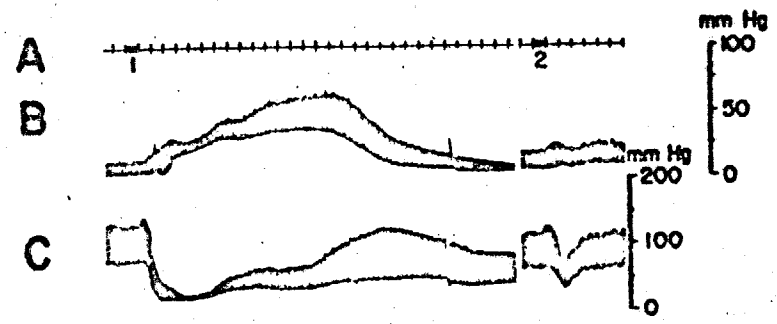

Fig. 3. *Effect of cottonseed oil emulsion on pressure in the pulmonary and carotid arteries.* Cat 3.3 kg, under chloralose anaesthesia with open thorax. A indicates 10-second intervals and injection times; B, the pulmonary artery; C, the carotid artery pressure. At 1 and 2, 0.5 ml/kg of a 10% cottonseed oil emulsion with phosphatides (1.2%) and pluronic (0.2%) as emulsifiers were injected.

2 minutes. Here too the effect declined with repeated injections and ultimately disappeared, although the injected doses were high.

The hypotensive action of these emulsions was substantially less after vagotomy, but in spite of this operation there was an unchanged rise of pressure in the pulmonary artery. This elevation in pressure showed a high degree of tachyphylaxis (fig. 5).

Direct experiments showed also that soyabean phosphatides caused a blood-pressure fall and apnoea, both of which effects exhibited marked tachyphylaxis. With repeated injections, however, the hypotensive action did not completely disappear (fig. 4), a result suggesting that two hypotensive factors were present here.

With cottonseed emulsions containing fatty acid, the first injections were sometimes accompanied by interference with the specific effects of

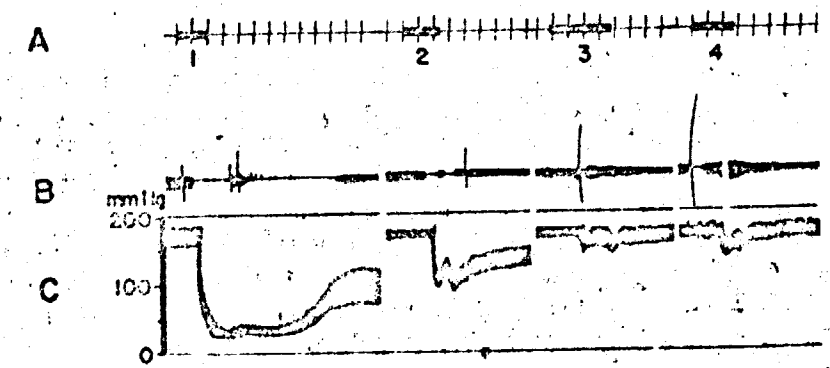

Fig. 4. *Effect of phosphatides on blood pressure and respiration.* Cat, 3.8 kg, under Dial ® anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, the blood pressure in mm Hg. At 1, 2, 3 and 4 4 ml/kg of 2.4% solution of soya bean phosphatides and 0.2% Pluronic F 68 were injected. The phosphatides have a hypotensive action characterized by tachyphylaxis, though a slight effect persists.

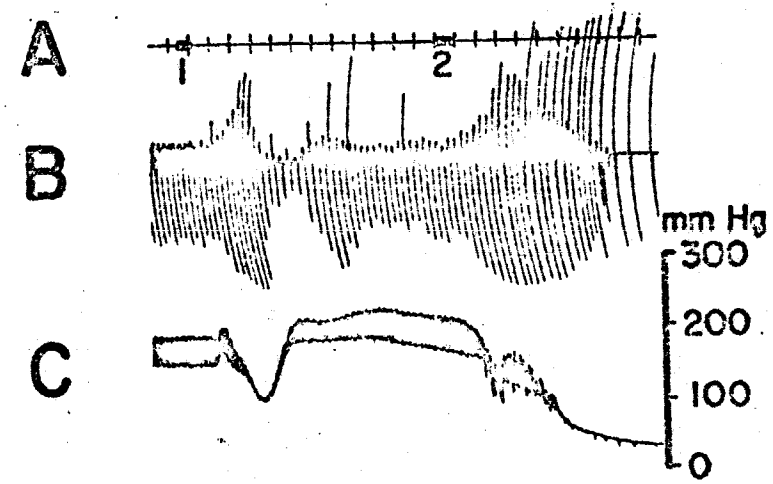

Fig. 6. *Effect of oleic acid emulsion on blood pressure and respiration.* Cat, 4.0 kg, under chloralose anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. One ml/kg of 10% oleic acid emulsion in cottonseed oil (10%) with soyabean phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at 1 and 2.

the contraction after 0.2 µg histamine. If the fatty acid molecule contained a greater or smaller number of carbon atoms than 10 or 11, the relaxing action on histamine-contracted intestine decreased; for instance, 150 mg of butyric acid was required for total inhibition. Comparison of undecylic acid with its unsaturated homologue, undecylenic acid, revealed no significant difference. The emulsion system alone without fatty acid is inert. No difference was detectable when the lower fatty acids were administered in aqueous solutions instead of in emulsions.

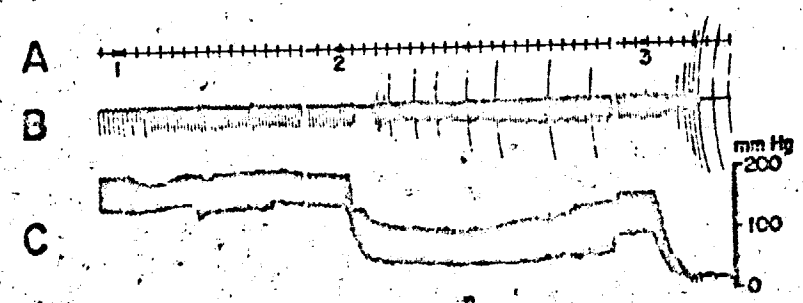

Fig. 7. *Effect of stearic acid emulsion on respiration and blood pressure.* Cat, 4.3 kg, under chloralose anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. At 1, 2 and 3, a 1% stearic acid emulsion with 10% cottonseed oil as carrier and phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at a dose of 0.2 ml/kg.

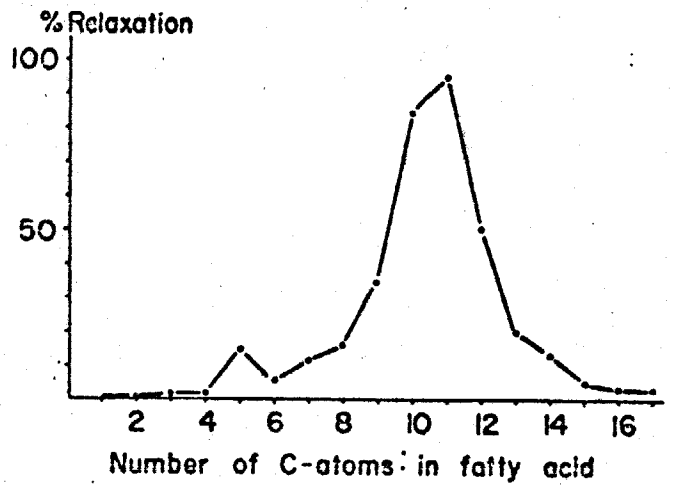

Fig. 8. *Relaxing action of fatty acids on guinea-pig small intestine contracted by histamine.* The fatty acids were administered in the form of emulsion. Abscissa indicates the number of carbon atoms in the fatty acid molecule. Ordinate shows percentage relaxation of guinea-pig intestine contracted by 0.2 µg histamine, after addition of 1.5 mg fatty acid to an organ bath containing 15 ml.

Discussion.

This investigation thus shows that several fatty acids are markedly toxic when administered intravenously. It is mainly the higher fatty acids that show this, (LD50 23 mg/kg) to the greatest extent. With a reduced number of carbon atoms in the molecule the toxicity diminishes, being least for caproic acid with six carbon atoms (LD50 1,724 mg/kg). When compared with the triglycerides of low molecular weight, the free fatty acids thus show the reverse behavior in toxicity; for the LD50 curve for triglycerides reveals a distinct minimum when the fatty acid component has five or six carbon atoms. Thus, hydrolysed fatty acids cannot be responsible for the acute toxic action of the triglycerides.

Our value for the LD50 of acetic acid (525 mg/kg) is closely consistent with that found by WELCH *et al.* (1944) – 380 mg sodium acetate per kg in mice. SAMSON, DAHL & DAHL (1956) described a hypnotic action of fatty acids of low molecular weight (C_2–C_{10}). There seems to be no direct correlation between hypnotic and toxic effects. The ED50 for hypnotic action decreases progressively with an increasing number of carbon atoms in the fatty acid molecule, in contrast to the LD50, the highest dose being that of caproic acid with six carbon atoms and the dose then falling with both increased and reduced numbers of carbon atoms.

The responses of respiration and blood pressure to the fatty acid emulsions differed from those to the triglycerides of low molecular weight

(WRETLIND 1957 a); in cats intravenous injection of more than 5 mg stearic acid or 50 mg oleic acid per kg was followed by apnoea and a fall in blood pressure as well as by convulsions with lethal outcome. Lower doses had a depressor action on blood pressure; the respiration sometimes being stimulated. The dose required for toxic action varied somewhat with the emulsifier employed. The effect on the circulatory system also differed with different emulsifiers. Stearic acid emulsions with phosphatides and Pluronic as emulsifiers raised the pulmonary arterial pressure substantially, and this elevation contributed to the fall of blood pressure in the systemic circulation. With hydrogenated phosphatides there was no demonstrable influence on pulmonary arterial pressure; on the other hand these emulsions had a pronounced effect on heart-lung preparations, so that their hypotensive action may be attributable to direct cardiac depression. Oleic acid emulsions that had been emulsified solely with phosphatides and Pluronic F68, without cottonseed oil, also had a hypotensive and toxic action on heart-lung preparations.

It is accordingly evident that the emulsified higher fatty acids studied had, on intravenous injection, a pronounced toxic action associated with a fall of blood pressure.

PELTIER (1956) determined the toxicity of some neutral fats and free fatty acids in non-emulsified form. He concluded that the toxic effects of fatty acid were caused by damage to the pulmonary capillaries, with extravasation into the alveolar spaces. JEFFERSON (1948) held a similar view. In our experiments on anaesthetized cats we occasionally observed progressive respiratory distress, and blood-tinged froth escaped from the tracheal cannula. At autopsy the lungs were oedematous. – It is worthy of note that the LD50 is more or less the same for non-emulsified oleic acid in rabbits (PELTIER 1956) and emulsified oleic acid in mice (250 mg/kg and 230/kg respectively). SCUDERI (1941) found that 0.33 ml of non-emulsified oleic acid per kg had a toxic action on the dog. The emulsified form had a lethal effect on anaesthetized cats in somewhat smaller doses, i.e., 50–100 mg/kg. However, total amounts substantially greater than 250 mg of emulsified oleic acid per kg can be given by repeatedly injecting sublethal doses.

The fatty acid emulsions had no stimulating action on *small intestine from the guinea pig* – a finding that accorded with results reported by GABR (1956). The emulsions could, however, relax histamine-contracted intestine. This effect was not correlated with their toxic action; rather the reverse, the relaxing effect being maximal for fatty acids with 10 or 11 carbon atoms and decreasing with either a smaller or greater number of carbon atoms in the molecule. Of interest in this connection is the finding of GANLEY *et al.* (1960) that higher fatty acids partially inhibited the

action of serotonin (5-hydroxytryptamine) and the effect in anaphylactic shock in mice.

As to the blood pressure fall that follows injection of emulsions of *cottonseed oil* or *triolein*, it is difficult to determine the mechanism exactly because of the pronounced tachyphylaxis.

If an anaesthetized cat is injected with 6 ml/kg 10% emulsion of cottonseed oil or triolein emulsified with 0.1 or 1.0% Pluronic F 68, a substantial fall in blood pressure as well as apnoea occur after about 10-60 seconds. The cat may even die after this first injection. The pulmonary arterial pressure sometimes rises as high as 100 mm Hg. Our results suggest that the hypotensive effect is elicited mainly via the vagus, for it is practically eliminated by vagotomy (fig. 5).

Since the responses of blood pressure can also be obtained with the soya bean phosphatides alone, it seems probable that the effects observed with the cottonseed oil and triolein emulsions were due to the presence of such phosphatides. That the unsaturated phosphatides were responsible also appears likely, inasmuch as there was no circulatory response when hydrogenated soya bean phosphatides were used as emulsifiers. The fact that cottonseed oil emulsions with Pluronic F 68 as the sole emulsifier produced similar effects on the blood pressure shows that this is not the only explanation. Our observations tend rather to suggest that the effects of intravenously administered fat emulsions are due to a number of factors, such as the properties of the fat, the emulsifiers, the surface tension, size of fat particles and the charge carried by them. On the basis of our present knowledge in this field, it is impossible to say finally which is the principal factor involved.

According to SEEVERS (1958), tachyphylaxis is probably due to specific "receptors" that may take up the active substance, thereby eliciting the pharmacological action. When no more of these receptors are available, the effect ceases. The active substance is subsequently released slowly, and not until the liberation is complete can an effect be once more obtained. In our experiments the recovery period was relatively long, at least four hours. It is not yet possible, however, to say where these receptors are situated or by what mechanism the fat emulsions block them.

Summary.

Pharmacological investigations of the effects of intravenously administered fatty acids of high molecular weight are handicapped by the fact that such acids are insoluble in water at neutral reaction. By utilizing fat particles in an emulsion as carriers of these fatty acids, it has been possible

in our investigation to study the toxicity and certain pharmacological effects of such acids when given intravenously. Although emulsions of the carrier fat are devoid of toxicity, they exhibit peculiar pharmacological effects characterized by pronounced tachyphylaxis. In this investigation the following observations were made.

The LD50 by intravenous injection into mice was determined for saturated *fatty acids* from C_2 to C_{18}. Of these, stearic acid emulsion showed the highest toxicity: LD50 23 mg/kg body weight. With a decreasing number of carbon atoms in the fatty acid molecule the toxicity fell, reaching a minimum for caproic acid: LD50 1,725 mg/kg. As the number of carbon atoms declined beyond that point, so did the toxicity rise again, the LD50 amounting to 525 mg/kg for acetic acid.

The LD50 for stearic acid was ten times less than for its unsaturated analogue, oleic acid. For the *cottonseed oil emulsion* employed as "carrier" the LD50 was indeterminable, being higher than 15 g cottonseed oil per kilogram.

The pharmacological effects of emulsions of *cottonseed oil and triolein* on respiration and blood pressure were investigated by intravenous injection into cats. With soya bean phosphatides and Pluronic F 68 as emulsifiers a blood pressure fall and apnoea as well as an elevation of the pulmonary arterial pressure were observed. These effects were also noted for fat emulsions emulsified solely with Pluronic F 68 and for unsaturated soya bean phosphatides alone. Emulsions with hydrogenated phosphatides, on the contrary, did not produce such effects. The action on the circulation and respiration showed marked tachyphylaxis.

The effects of higher *fatty acid emulsions on circulation and respiration* were characterized by a fall in blood pressure as well as hyperpnoea or apnoea. Such emulsions also had a negatively inotropic effect on the heart.

Fatty acid emulsions had no direct action on *small intestine* from the guinea-pig, but inhibited contractions elicited by histamine.

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and other sterols and steroids are also present in smaller amounts. Under conditions such as fasting, or in untreated diabetes, where lipid must be metabolized in greater than normal amounts because of the unavailability or inability of carbohydrate to supply its normal quota of the caloric requirement, the lipid content of the liver is increased. In various abnormal states the lipid content of the liver may increase to 30 or 40 per cent, giving rise to the condition termed "fatty liver." The synthesis and oxidation of fatty acids take place readily and rapidly in the liver. Phospholipid turnover is also rapid. Cholesterol, cholesterol ester, and bile salt syntheses occur in the liver. In the liver the carbon chain of fatty acids can be lengthened or shortened, saturated or desaturated.

Metabolism of Glycerol. On hydrolysis, neutral fats yield glycerol and fatty acids. As noted earlier, glycerol can be metabolized via conversion to glycerol phosphate and dihydroxyacetone phosphate, which enter the glycolytic pathway and may either be converted to glycogen or oxidized to CO_2 and H_2O. By the reverse process, the glycerol portion of lipid molecules may be formed from carbohydrate.

Metabolism of Fatty Acids. The major pathway whereby fatty acids are oxidized in the animal body is by the process known as β-oxidation. By this process the fatty acid chain is oxidized at the carbon atom β to the carboxyl group, and is subsequently split into acetyl coenzyme A and a chain two carbons shorter in length. The process is repeated until the entire molecule has been broken down into acetyl CoA.

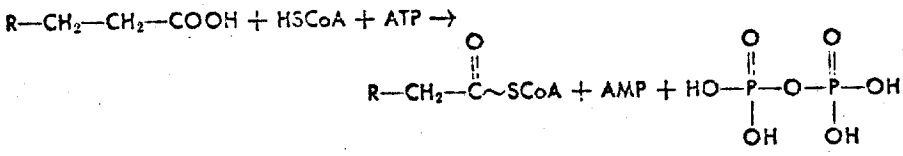
The first biological evidence for β-oxidation was afforded by the experiments of Knoop. This investigator found that on feeding animals fatty acids of varying chain length, but in each case with the ω-carbon (the one farthest removed from the carboxyl carbon) attached to a phenyl group, there could be isolated from the urine either benzoic acid or phenylacetic acid, in the form of the conjugated derivatives, hippuric acid and phenaceturic acid, respectively. Benzoic acid was obtained when the fatty acid chain contained three or five carbon atoms; phenylacetic acid when it contained two (i.e., phenylacetic acid itself) or four carbon atoms. These results led Knoop to conclude that the carbon atoms could not be removed one by one during oxidative breakdown of the fatty acid, but must come off in pairs, i.e., by oxidative removal at the β-carbon, since if the carbons came off one at a time, then the two-carbon acid would be converted to a one-carbon acid and benzoic acid would have been excreted; phenylacetic acid, however, was actually found. In the same manner, if one carbon had been removed from the three-carbon acid producing a two-carbon acid, phenylacetic acid should have been excreted. Since, in fact, benzoic acid was excreted, two carbons must have been removed as a unit, and not one at a time.

The mechanism of β-oxidation and the nature of the two-carbon fragment produced in the process has been the subject of prolonged and intensive study. A major difficulty, since overcome, was the inability to obtain cell-free preparations from animal tissues which were capable of oxidizing fatty acids and were susceptible to experimental study. Early studies were also made difficult because the role of coenzyme A was not known and it was not recognized that after the initial step all further reactions were reactions not of a fatty acid, but of fatty acid acyl coenzyme

A derivatives. Much of the more recent work establishing the mechanism of fatty acid oxidation has been carried out by groups headed by Green,[24] Lynen,[25] and Kennedy.[26]

Five reactions are involved in the β-oxidation process, as described below. These reactions take place in the cell mitochondria.

1. Activation. The initial reaction consists in the formation of an acyl coenzyme A derivative from the free fatty acid.



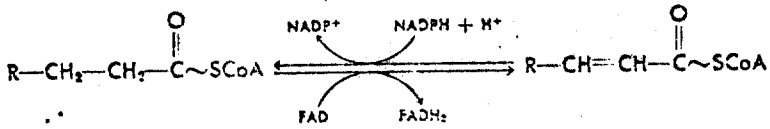
At least three activating enzymes (thiokinases) exist. One acts on acetic and propionic acid, the second acts on acids having four to twelve carbon atoms and the third on acids having more than twelve carbon atoms.

Butyric and acetoacetic acids may also be activated by a reaction with succinyl CoA, in the presence of a thiophorase.

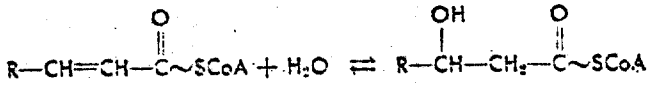
$$\text{Butyrate} + \text{Succinyl CoA} \rightleftarrows \text{Butyryl CoA} + \text{Succinate}$$

The thiokinases require a supply of ATP and CoA, while the thiophorases require NAD, as in the reaction by which α-ketoglutarate is converted to succinyl CoA. They do not require ATP. Succinyl CoA can also be formed from succinate and GTP by a thiokinase.

2. Desaturation. Once the fatty acid has been activated, it can be dehydrogenated in the α, β position by acyl dehydrogenases. These enzymes contain a flavoprotein, flavin adenine nucleotide, and they show specificity in relation to the chain length of the fatty acid. The reaction is not reversible by the same enzyme but is reversible in the presence of reductases which require NADPH.



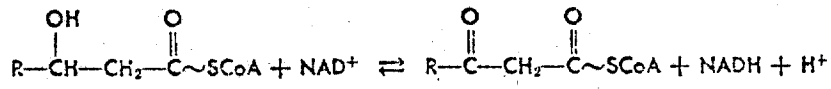
3. Hydration. In the next reaction, a molecule of water is added across the double bond, in the presence of enoyl hydrases, to form a β-hydroxyacyl CoA derivative.



4. Oxidation. In the presence of β-hydroxyacyl dehydrogenases and NAD^+, the hydroxyl group of the β-hydroxyacyl CoA derivative is oxi-

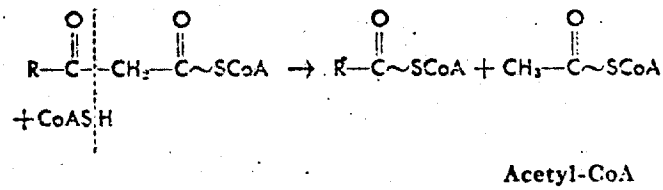
[24] Green: *Biol. Rev. Cambridge Phil. Soc.*, 29, 330 (1954).
[25] Lynen: *Ann. Rev. Biochem.*, 24, 653 (1955).
[26] Kennedy: *Ann. Rev. Biochem.*, 26, 119 (1957).

dized to a keto group



The NADH and the $FADH_2$ formed in the dehydrogenation steps are oxidized through the hydrogen transport system and provide part of the energy, in the form of ATP, which is obtained from fatty acid oxidation.

5. Thiolytic Cleavage. The final step in the process of β-oxidation is cleavage of the β-keto derivative by a molecule of coenzyme A. The reaction is similar to hydrolysis but involves the sulfhydryl group of CoA instead of water and is called thiolysis. The enzymes are called thiolases and appear to be nonspecific, covering β-ketoacyl CoA esters from C_4 to C_{18}.



The products of the reaction are a mole of acetyl CoA and a mole of an activated fatty acid which is two carbons shorter than the fatty acid at the start. The activated fatty acid can be further degraded by a repetition of the process, starting at the second reaction, since activation is not necessary. By successive repetitions of the process the entire fatty acid chain can be converted to acetyl CoA. The acetyl CoA mixes with the acetyl CoA pool derived from carbohydrate and amino acid metabolism and participates in many biological processes in addition to those already described. Further aspects of acetyl CoA metabolism are discussed below.

Ketosis. Under normal metabolic conditions most of the acetyl CoA formed from the oxidation of fatty acids, pyruvate, and other sources is condensed with oxalacetic acid and oxidized through the tricarboxylic acid cycle. However, in circumstances when the metabolism of carbohydrates is impaired or operating at a low level, such as in diabetes mellitus, starvation, or prolonged subsistence on a low carbohydrate diet, the fate of acetyl CoA is altered for two reasons: (1) the oxalacetate available to condense with acetyl CoA is in limited supply and, (2) a much greater proportion of the body's energy needs is being supplied by the oxidation of fatty acids, leading to the production of acetyl CoA in greater than normal amounts. Because of this combination of circumstances (large amounts of acetyl CoA and small amounts of oxalacetate), acetyl CoA metabolism proceeds to a greater extent than normally via a different route. This consists of the condensation of two moles of acetyl CoA to form acetoacetyl CoA. Through the action of deacylases in the liver, acetoacetyl CoA is hydrolyzed to coenzyme A and acetoacetic acid. Acetoacetic acid may be reduced to β-hydroxybutyric acid

Other Methods. Other tests for carbohydrate tolerance include the insulin,[125] tolbutamide,[126] epinephrine,[125] and the glucagon tolerance tests.[125]

DETERMINATION OF FATTY ACIDS

Introduction. The fatty acids of blood are present largely as esters in the form of (*a*) neutral fat, (*b*) phospholipids, (*c*) cholesterol esters; there may be a small amount of free fatty acid (i.e., soaps) also present. The total fatty acids of whole blood are distributed approximately evenly between cells and plasma; this even distribution apparently simply reflects the fact that while phospholipids for example are much more abundant in the cells than in the plasma, the reverse is true for esterified cholesterol, which is apparently absent from the cells. Total fatty acid content is usually determined by saponification and titration of the liberated fatty acids.

Method of Stoddard and Drury[127]

Principle. The blood is extracted with alcohol-ether, the extract saponified, the fatty acids separated, filtered, washed, dissolved in alcohol, and titrated with thymol blue as indicator.

Procedure. EXTRACTION. Five ml of whole blood, plasma, or serum is introduced gradually into a 100-ml volumetric flask containing about 75 ml of a mixture of 95 per cent alcohol and redistilled ether (3:1). The flask is immersed in boiling water and rotated frequently and vigorously (to prevent superheating) until boiling begins, then cooled to room temperature, made up to volume with alcohol-ether, mixed, and filtered through fat-free filter paper.

SAPONIFICATION. To a 100-ml beaker containing a few grains of coarse sand (previously boiled with acid, washed, dried, and extracted with ether) add gradually, while evaporating on a water bath, 75 ml of the filtrate. The temperature should be low enough to avoid perceptible boiling but may be raised after the ether has evaporated. Evaporate to a volume of about 30 ml. Add 0.1 ml of saturated CO_2-free NaOH,[128] mix, add a few grains more of sand, cover with a watch glass, and boil gently for 20 to 30 minutes (to saponify).

SEPARATION AND WASHING. Remove the watch glass, drop in a small piece of litmus paper, make acid with 30 per cent HCl; then run back to alkalinity with 10 per cent NaOH (in order to avoid an excess of alkali on evaporating to dryness). Evaporate to dryness (in order to get rid of all alcohol). Add 15 ml of water, heat on the steam bath, and stir to dissolve the soaps. While hot, add a drop of thymol blue indicator and make acid (faint pink) with 30 per cent HCl. Set the beaker for 10 minutes in cold water, then swirl almost continuously for five minutes to produce a better separation of the fatty acids.

Filters are previously prepared as follows: Use a Gooch crucible, smallest size (top 28 mm, bottom 18 mm in diameter). Set the crucible in a rubber holder which fits over a 500-ml suction flask. A paper-pulp suspension is made by shaking up a piece of soft filter paper in 300 to 400 ml of distilled water. Shake vigorously and immediately pour some into the crucible while there is a strong suction on. Repeat until a layer about 1 mm thick is formed. Tamp the layer down carefully all over with the end of a glass rod. Allow the larger masses of filter pulp fibers in the suspension to settle out, and pour on successive amounts of the thin upper suspension of isolated shreds, keeping a strong suction on and

[125] Fraser, Albright, and Smith: *J. Clin. Endocrinol.*, **1**, 298 (1941).
[126] Unger and Madison: *Diabetes*, **7**, 455 (1958).
[127] Stoddard and Drury: *J. Biol. Chem.*, **84**, 741 (1929). This method has been critically evaluated by Man and Gildea: *J. Biol. Chem.*, **99**, 43 (1933).
[128] See Appendix I.

J. Lab. & Clin. Med., 14(7): 630-635 (1929)

THE SEDIMENTATION OF RED BLOOD CELLS*

By C. E. Reyner, New Haven, Conn.

SINCE Fahraeus in 1918[8] revived the old observation that the red blood cells in blood obtained from patients with certain pathologic conditions settled with much greater speed than those secured from normal persons, a great literature, most of which has appeared in European journals, has accumulated upon the subject. Considerable enthusiasm has been displayed by numerous writers, while others, particularly in this country, have exhibited greater conservatism as to the value of the test in diagnosis and prognosis. Although the test appears to have considerable value in the study of certain pathologic conditions, particularly pulmonary tuberculosis, the clinical application is not the only feature to receive consideration. Interest in the test has involved primarily the mechanism whereby the rate of sedimentation is under certain conditions so markedly increased, and it is in connection with this aspect of the reaction that the following data are presented.

Almost as many theories, explaining the acceleration of the sedimentation rate in certain diseases, have been proposed as there have been authors who have reported upon the reaction. Fahraeus thought that it was due to a change in the electric charge of the red blood cells causing a loss of their repelling force and bringing about the production and agglutinates which settled more rapidly. However, he was unable to establish that theory by experimental evidence and later suggested that viscosity may be a factor. Others such as Jevons,[7] Hueber,[9] Risse,[22] and Pohle[24] advanced ideas similar to the theory of electric changes of Fahraeus. Northrup and Freund,[18] and

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Oliver and Bernard[19] have shown that the stability of the red cell suspension is largely dependent upon the difference in potential between the cells and the medium in which they are suspended. If the potential difference falls to a certain point the red cells agglutinate. However, Pender[20] points out that calcium chloride and magnesium chloride reduce the potential difference, but the red cells do not agglutinate. A number, among whom may be mentioned Katz,[10] Plaut,[23] Starlinger and Frosch,[24] Plass and Rourke,[22] Abderhalden,[1] Levinson,[12] Linzenmeier,[14] Buscher,[3] Westergren,[26] and Alexander[2] believe that the increased sedimentation rate is due to the increased content of fibrinogen in the blood. Newham,[17] however, found that the fibrinogen content was normal in 14 cases which showed accelerated sedimentation. Schmitz,[33] Sachs,[31] Salomon,[32] and Fischel[5] thought that an alteration in the globulin-albumin ratio with an increase in globulin caused the increase in the rate of sedimentation. Wichels[37] favored the destruction of red blood cells as being the primary factor. An increase in the blood cholesterol as a cause for the acceleration was suggested by Kurten,[11] but this idea was opposed by Newham[17] who showed that the blood cholesterol was normal in 15 cases which exhibited accelerated sedimentation rates. Leendertz[13] believed that the presence of antibodies was the contributory factor, while Lohr,[15] and Herzfeld and Schinz[7] suggested that an alteration in the viscosity may cause the phenomenon. Others believe that the explanation does not lie in one factor alone, but that several factors collectively contribute to it. Morriss[16] suggests that the surface tension, capillary attraction and a difference in electric charges cause the acceleration. Pribram and Klein[26] think that increased viscosity and blood cholesterol may explain the reaction, while Popper and Kreindler[25] suggest that an increase of both fibrinogen and globulin offers the explanation. Cooper[4] believes that an increase in fibrinogen, cholesterol and globulin is the answer, and Petschacher[21] offers an alteration in viscosity and a high globulin content in the blood as a possible explanation. Rubin[30] thinks that the entire structure of the blood is altered so as to effect the characteristic acceleration in the rate of sedimentation. Puxeddu[27] provides evidence showing that the phenomenon is resident solely in the blood plasma while Newham[17] affirms that it is primarily located in the red blood cells. Wells, DeWitt and Long[35] have pointed out that after severe hemorrhages, the blood shows an increase in surface tension, and in tuberculosis the return to normal will be slower in proportion to the progress of the disease. Other investigators have observed changes in the total protein content, in lecithin, lipase, and phosphorus, as well as alterations in the size, number and hemoglobin content of the red blood cells of blood that shows an accelerated rate of sedimentation.

Manifestly, in view of such discordant opinions, the basic cause for accelerated sedimentation is obscure.

In connection with work which has been reported on the mode of action of formaldehyde in complement fixation systems,[28] it was observed that bacterial suspensions to which formaldehyde had been added were more difficult to throw down in the centrifuge than were those which had not been so

treated. Investigation of this point revealed the fact that the addition of formaldehyde increased the surface tension and, apparently, in that way sedimentation of the bacteria was made more difficult. The du Nouy surface tensiometer was employed and, in terms of dynes, the bacterial suspension showed a surface tension of 60 while the suspension with formaldehyde added to a concentration of 1:50 gave a reading of 67 dynes. These facts suggested the application of the surface tension principle to a study of the sedimentation of red blood cells.

The addition of formaldehyde to whole blood, to blood serum, to blood plasma, and to red corpuscles suspensions elevated the surface tension by about 5 dynes in each case even though as low a concentration as 1:250 was employed. Sedimentation tests were performed after a method which has been described by Morriss.[16] In certain tubes varying amounts of formaldehyde were mixed with the blood which was being tested. Upon making readings at regular intervals, it was evident that formaldehyde altered the rate of sedimentation of the red cells. A fairly strong concentration, 1:250, slowed the rate of sedimentation markedly, and weaker concentrations of formaldehyde permitted increasing rates of sedimentation up to that which was obtained when no formaldehyde was included in the test.

In view of the possibility that changes other than an altered surface tension might result from the addition of formaldehyde, a surface tension reducent, sodium oleate, was utilized and a similar series of sedimentation tests were performed in which the reducent was mixed with the blood before being drawn up in the pipettes. In this case it was assumed that the only changes effected by the sodium oleate were physical in character. The sedimentation rate of red blood cells was very markedly reduced by the addition of minute amounts of this reducent, so markedly, in fact, that after two hours, when the control showed a sedimentation of 10 mm., the sodium oleate tubes showed none whatever. This very remarkable alteration in the rate of sedimentation was interpreted as being due to the reduced surface tension. By altering the surface tension with this reducent almost any degree of sedimentation from zero to that exhibited by the untreated blood may be obtained.

Working on the assumption that variations in the sedimentation rate of red blood cells are due to alterations in the surface tension of the plasma, bloods were collected from patients with pulmonary tuberculosis, and sedimentation tests were conducted with and without sodium oleate, as seen in Table I.

In every case the sedimentation was reduced by sodium oleate to 2 mm. or less in two hours, whereas without the reducent the sedimentation was as great as 57 mm. However, the protective influence against sedimentation, afforded by the sodium oleate, appeared to be lost, at least partially, after twelve hours, for a number of specimens of blood, when close to that time interval, suddenly began to sediment rapidly. For example, in the interval between two and twelve hours, in one instance the cells settled from 1 mm. to 60 mm.; in another, from 0 mm. to 45; and in still another, from 1 mm. to 48 mm. At the twenty-four-hour period this phenomenon was even more

marked, so that at that time the readings were nearly the same with and without sodium oleate. The explanation of this observation has not yet been obtained.

It is interesting to note in passing that the case of pulmonary tuberculosis, classified as minimal A and that classified as moderately advanced A, exhibited such slow rates of sedimentation. (In the Table they are Numbers 4 and 3.)

TABLE I

TYPICAL RESULTS OF SEDIMENTATION TESTS PERFORMED UPON THE BLOODS FROM TUBERCULOUS PATIENTS AND NORMAL CASES, COMPARED WITH RESULTS OBTAINED WHEN SODIUM OLEATE WAS ADDED IN THE CONCENTRATION OF 1:2000

SPECIMEN NUMBER		SEDIMENTATION IN MM. PER 100 MM. HOURS					CLINICAL DIAGNOSIS
		0.5	1.0	2.0	12.0	24.0	
1	Native blood	3	15	30	46	47	Pulmonary tuberculosis, far advanced A.
	+ Sod. oleate	0	0	0	1	4	
2	Native blood	8	24	38	47	49	Pulmonary tuberculosis, far advanced B.
	+ Sod. oleate	0	0	0	1	43	
3	Native blood	0	0	4	5	12	Pulmonary tuberculosis, moderately advanced A.
	+ Sod. oleate	0	0	0	1	3	
4	Native blood	1	1	2	12	22	Pulmonary tuberculosis, minimal A.
	+ Sod. oleate	0	0	0	2	3	
5	Native blood	9	24	39	55	57	Pulmonary tuberculosis, far advanced B.
	+ Sod. oleate	0	0	0	4	29	
6	Native blood	22	42	57	63	64	Pulmonary tuberculosis, far advanced C.
	+ Sod. oleate	0	0	1	60	60	
7	Native blood	14	32	45	57	58	Pulmonary tuberculosis, far advanced B.
	+ Sod. oleate	0	0	0	5	16	
8	Native blood	4	9	19	40	44	Pulmonary tuberculosis, far advanced A.
	+ Sod. oleate	0	0	1	3	11	
9	Native blood	8	22	41	54	56	Pulmonary tuberculosis, far advanced A.
	+ Sod. oleate	0	0	1	25	45	
10	Native blood	0	1	2	3	4	Normal.
	+ Sod. oleate	0	0	0	0	1	
11	Native blood	20	36	48	55	55	Pulmonary tuberculosis, far advanced B.
	+ Sod. oleate	0	0	0	45	48	
12	Native blood	5	11	26	53	55	Pulmonary tuberculosis, far advanced C.
	+ Sod. oleate	0	0	1	18	48	
13	Native blood	0	2	6	20	30	Normal
	Sod. oleate	0	0	0	0	1	

It is evident, therefore, that a minute amount of sodium oleate, through its action as a surface tension reducent, very markedly decreases the rate of sedimentation of red blood cells in normal as well as in pathologic cases. In a number of instances the plasma was replaced by serum and the same rate of sedimentation was obtained in each case, which suggests that the fibrinogen factor in accelerating sedimentation is negligible.

Studies have been made upon specimens of blood from patients with pulmonary tuberculosis in varying stages, with the du Nouy surface tensiometer, but without detecting any very marked alteration in the surface tension. It is entirely possible that the changes are those of an interfacial character and not readily demonstrable by the apparatus. However, upon determining the surface tension of a specimen of plasma, it was found, for example, to be 61.7; with added sodium oleate, 52.4; and with added formaldehyde, 63.6. Nevertheless, there does not appear to be any other interpretation of the effect of sodium oleate upon the rate of sedimentation than that of an alteration of surface tension and it appears very probable that, since this reducent slows the sedimentation of blood from tuberculous patients through its effect in lowering the surface tension, the explanation of the accelerated sedimentation lies in an increased surface or interfacial tension. The cause of the alterations of the surface tension in the blood of tuberculous patients is problematical.

CONCLUSIONS

1. The surface tension of blood is increased by the addition of formaldehyde and is markedly decreased by the addition of sodium oleate.

2. The rate of sedimentation of red blood cells is greatly diminished by sodium oleate in normal bloods as well as in bloods obtained from patients with pulmonary tuberculosis, suggesting that the acceleration in rate of sedimentation in tuberculosis and other infectious diseases is explainable upon the basis of surface tension.

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INHIBITION OF GASTRIC SECRETION IN THE RAT WITH SODIUM OLEATE

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THE INHIBITORY EFFECT of large amounts of fats on gastric secretion has been known for almost a century [4]. Experiments have indicated that the effect may be due to fat in contact with the duodenal mucosa [3, 5, 9]. Further experimentation using the products of lypolysis (fatty acids and soaps) have been carried out with results indicating that these products have greater inhibitory effects on gastric mobility and secretion [7, 8]. More recently [1, 2] intravenous emulsions of fat have been shown to decrease gastric secretions. Most of these previous experiments have used natural oils (mainly olive oil, and/or the enzymic degradation products of these oils). Olive oil is composed of mixed glycerides of oleic acid 83.5%, palmitive acid 9.4%, linoleic acid 4.0%, stearic acid 2.0%, and arachidic acid 0.9%. Minor constituents include squalene, phytosterol, and tocopherols [6]. It is the purpose of this experiment to eliminate some of the variables inherent in the use of impure natural products. In addition, we have attempted to elucidate, to some small extent, the possible pathway of this phenomenon.

Toward this end, we have substituted, for the impure products, purified fatty acids in much smaller quantities as experimental inhibitors. In addition, we have used two different routes of administration, intravenous and intraperitoneal.

Materials

Oleic acid, linoleic acid, stearic acid, and propionic acid were used as 0.1 N solutions, adjusted to pH 8. Oleic acid (0.2 N), adjusted with NaOH, to pH 8 was used in one test group. Distilled water and normal saline were used in the controls. Male Wistar rats weighing between 100 and 200 gm. were used as test animals.

Method

The volume of gastric secretions was determined in accordance with the method of Shay (10). The animals were purchased from a reputable supply house and kept for 2 weeks in group cages to become accustomed to their environment. Solutions were administered intraperitoneally and intravenously after fasting the animals for 48 hours allowing H_2O ad libitum. In those instances in which the test solutions were administered intraperitoneally, this was done through the abdominal wound made for the pyloric ligation just prior to its closure. In those instances in which the solutions were administered intravenously, a cutdown was made in the thigh, the femoral vein exposed and injected after closure of the ab-

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Table 1. Results of Intraperitoneal Administration of 0.25 cc. of Test Substances

Substance	Number of Rats	Average ml. Secretion 100 gm.	S.D.	S.E.	P
Normal saline	24	3.6	0.833	0.155	—
Distilled water	31	3.1	0.971	0.175	> 0.050
Nothing	26	3.7	0.922	0.182	> 0.050
0.1 N Na propionate	22	3.3	0.851	0.182	> 0.200
0.1 N Na stearate	27	3.6	0.726	0.139	> 0.500
0.1 N Na linoleate	28	3.1	0.934	0.176	> 0.500
0.1 N Na oleate[a]	36	2.0	0.770	0.130	< 0.001
0.1 N Na oleate[a]	29	1.9	0.987	0.183	< 0.001
0.2 N Na oleate[a]	24	1.7	0.943	0.178	< 0.001

[a]The differences between these three groups are not significant.

Table 2. Results of Intravenous Administration of 0.25 cc. of Test Substances

Substance	Number of Rats	Average ml. Secretion 100 gm.	S.D.	S.E.	P
Normal saline	26	3.0	0.845	0.167	—
0.1 N Na oleate[a]	25	1.9	0.698	0.140	< 0.001
0.2 N Na oleate[a]	25	1.5	0.914	0.183	< 0.001

[a]The difference is not significant.

dominal wound. The animals were then sacrificed after a 4-hour period, again according to the method of Shay.

Volumes were reported as cubic centimeters per centigram of weight. The results were then analyzed statistically and the criteria for significance was a P value of 0.01 or less when compared to a control treated with normal saline.

RESULTS

Significant suppression of volume of gastric secretion by intraperitoneal administration of 0.1 N sodium oleate as compared to controls treated by intraperitoneal administration of normal saline occurred (Table 1). Intravenous administration of sodium oleate also significantly reduced the volume of gastric secretion as compared to the control of normal saline (Table 2). Intraperitoneal sodium propionate, sodium linoleate, and sodium stearate had no effect, giving results not significantly different from the controls.

DISCUSSION AND CONCLUSIONS

It appears that oleic acid, as its sodium salt, decreases the volume of gastric secretion in rats by one-third over a 4-hour test period. It further appears that the effect is independent of the contact of this fatty acid with the duodenal mucosa, but rather is a function of its presence in the bloodstream. Others have shown that olive oil in the duodenum without bile salts, does not inhibit gastric secretion. It is, therefore, reasonable to conclude that secretory inhibition by olive oil in the duodenum is caused by the products of lipolysis, Na oleate, after it has been absorbed and is in the bloodstream. The fact that neither linoleic acid (C_{18} doubly unsaturated), stearic acid (C_{18} saturated), or propionic acid (C_3 saturated) suppressed gastric secretion

indicates that this may be a specific pharmacological effect of oleic acid. The ability of linoleic acid (C_{18} triply unsaturated) to affect secretion has not been evaluated. Current preliminary studies indicate a suppression of secretion of HCl with decreased concentration of HCl using slightly larger quantities of Na oleate.

SUMMARY

The effect on gastric secretion in the rat by small quantities of sodium oleate (7.5 and 15.0 mg.) has been investigated. It was found that intravenous or intraperitoneal administration of small amounts (0.25 cc. of 0.1 N solutions) decreased gastric secretion by one-third over a 4-hour period. Sodium propionate, sodium stearate, sodium linoleate, other fatty acids, and normal saline did not inhibit gastric secretion under identical conditions. We conclude, therefore, that Na oleate may have a specific pharmacologic effect on gastric secretion in the rat. Furthermore, this action is not predicated upon contact with the duodenal mucosa but rather a function of its presence in the bloodstream.

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Influence of Unsaturation on Fibrinolytic Activity of Salts of Fatty Acids. (32416)

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Sodium salts of long chain saturated fatty acids have been reported to accelerate blood clotting and artificial thrombus formation *in vitro*(1,2) and to cause massive thrombosis and death in dogs(3,4). Unsaturated fatty acids are relatively inactive. We have found recently that sodium salts of long chain fatty acids can induce lysis of bovine fibrin films, and in this case it appeared that the unsaturated acids were more active than the saturated. No lysis occurred on heated plates. These preliminary findings are confirmed and extended in the present report.

Materials and methods. Fibrin films were prepared by dissolving Armour bovine fibrinogen (2.5 mg/ml) and Parke, Davis bovine thrombin, topical (50 NIH units/ml) in sodium borate buffer pH 7.7(5). Fibrinogen solutions were sterilized by filtration and 10 ml volumes were added to 8.5 cm Petri dishes. With the plates on a level surface, 0.5 ml of thrombin solution was added dropwise to the fibrinogen while the mixture was gently swirled to assure thorough distribution. Highly purified fatty acids were purchased from Applied Science Laboratories, State College, Pa. They were converted to their sodium or potassium salts by gentle warming with sodium (test no. 1) or potassium (tests 2-5) hydroxide and were adjusted to pH 8.1 with HCl. Two-fold dilutions of the fatty acids in sodium borate buffer were placed on fibrin films in 0.02 ml amounts. The lowest concentration which caused liquefaction sufficient for complete perforation of the fibrin film after incubation at 37°C for at least 4 hours was taken as the end point. (Identical results were obtained with oleate when the system was adjusted to pH 7.4).

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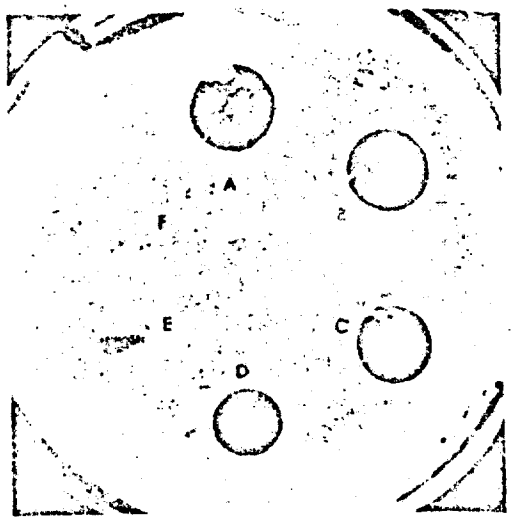

FIG. 1. Lysis of unheated bovine fibrin by sodium myristate. (A) 20 mM, (B) 10 mM, (C) 5mM, (D) 2.5 mM, (E) 1.25 mM, (F) 0.63 mM.

Results and discussion. Fig. 1 shows the activity of 2-fold dilutions of sodium myristate. At concentrations of 20, 10, 5, 2.5 and 1.25 mM, 0.02 ml amounts placed on the fibrin film caused complete perforation of the film. Liquefaction caused by 0.63 mM concentration was insufficient to perforate the film.

Saturated fatty acids having 16, 18, 20, 22 and 24 carbons were compared with acids with one unsaturated bond (Table I). Palmitic and palmitoleic acids appear to be

TABLE I. Influence of Unsaturation on Fibrinolytic Activity.

Fatty acid		Active concentration, mM — Test No. 1	2	3	4	5
Palmitate	C16:0		5	.3	.3	
Palmitoleate	C16:1		.5	.3	.3	
Stearate	C18:0		.6	1	1	
Oleate	C18:1	.2	.06	.2	.08	
Elaidate	C18:1		.06	.2	.2	
Arachidate	C20:0		1	5	1	
Eicosenoate	C20:1					.08
Behenate	C22:0		>10	>10	>10	
Erucate	C22:1		.03	.02	.01	
Lignocerate	C24:0					>10
Nervonate	C24:1					.08

equally active. Presence of one unsaturated bond in C18 and C20 acids increases activity roughly 10-fold. Presence of one unsaturated bond in C22 and C24 acids increases activity well over 100-fold.

The data in Table II suggest that although one unsaturated bond increases activity, further unsaturation may decrease it.

In Table III it can be seen that saturated fatty acids of chain lengths 12 to 20 are fibrinolytic.

In the present study, the ability of certain long chain fatty acids to cause fibrinolysis has been studied only on bovine fibrin films. Any conclusions concerning the importance of these findings will of course require further study in other assay systems. In his investigations of nonenzymatic activation of human plasminogen by various organic compounds, von Kaulla(6) found certain short chain saturated fatty acids to be active; tests with unsaturated and long chain fatty acids were not reported. Although, in our work, the inactivity of long chain fatty acids on heated fibrin films suggests that the lysis of unheated films may be caused by activation

TABLE II. Influence of Polyunsaturation.

Fatty acid		Active concentration, mM — Test No. 1	2	3	4	5
Oleate	C18:1	.2	.06	.2	.08	
Elaidate	C18:1		.06	.2	.2	
Linoleate	C18:2	.2	.5	.3	10	
Linolenate	C18:3		.1	.6	.6	
Eicosenoate	C20:1					.08
Arachidonate	C20:4			1	1	

TABLE III. Influence of Chain Length on Activity of Saturated Fatty Acid Salts.

Fatty acid		Active concentration, mM — Test No. 1	2	3	4
Laurate	C12	.5	10	3	10
Myristate	C14	1	.6	3	1
Pentadecanoate	C15		1	6	3
Palmitate	C16		5	.3	.3
Stearate	C18		.6	1	1
Nonadecanoate	C19		.6	3	1
Arachidate	C20		1	5	1
Behenate	C22		>10	>10	>10

of plasminogen, we have not ruled out a direct nonenzymatic dissolution of fibrin as in the case of surfactants(7).

Active interest in fibrinolysis caused by fatty acids seems justified because fatty acids are reported to cause significant effects on blood clotting systems *in vivo*. For example, a lethal intravenous infusion of fatty acids in dogs is associated with massive thrombosis if stearic acid is injected(3), and with pulmonary edema and hemorrhage in the case of oleic acid(8). In man, ingestion of linolenic acid can reduce elevated thrombotic tendency (9).

A role of plasminogen activator in preventing formation of thrombi was suggested to Gans(10) by the finding that increased plasminogen activator occurs as early as does hypercoagulability following intravenous administration of endotoxin to dogs. Man and animals have great capacity for effecting rapid changes physiologically in plasma concentrations of fatty acids, fatty acid compounds and fatty acid binding materials as

well as of both fibrinolytic and thrombotic inhibitors. Various changes also occur in pathologic states. It seems reasonable to suspect a possible role of fatty acids in regulation of fibrin formation and digestion.

Summary. We have found that sodium or potassium salts of some long chain fatty acids induce fibrinolytic activity on unheated bovine fibrin films. The influence of chain length and unsaturation on ability to induce fibrinolysis has been studied. Unsaturated fatty acids seem to be more active than the saturated compounds. The presence of one unsaturated bond appears to make little difference in activity of C16 fatty acids. In C18 and C20 acids, it increases activity roughly 10-fold. In C22 and C24 acids, it increases activity more than 100-fold. A possible role of fatty acids in regulation of fibrin formation and digestion is suggested.

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Vol. 2, part B
F.J. Welcher, Editor
Year 1963
D. Van Nostrand Co. Inc.
Princeton, N.J.

SOAP

(Dissolved)

Sodium soaps may be present in oils which have been treated with sodium hydroxide or sodium carbonate as in the alkali refining process. Traces of soaps are difficult to remove in processing and they are not easy to determine analytically. Soap has been determined for many years by simply ashing the sample and then weighing or titrating the residue with standard acid. This method is satisfactory for determining soap content of some significant quantity but it is not satisfactory

for trace amounts. A titrimetric method has been used for small quantities with apparent success.

A method recently adopted by the American Oil Chemists' Society is based on comparison of the conductivity of a water extract of the sample with the conductivity of aqueous solutions of known concentrations of sodium oleate.[7]

ASHING METHOD

Procedure.—Incinerate 75–100 g. of sample following the procedure previously described for the determination of ash. Add 50 ml. of cold, boiled distilled water to the ash and titrate the solution with 0.02 *N* acid using methyl orange as the indicator.

$$\% \text{ Soap as sodium oleate} = \frac{\text{ml. } 0.02\ N \text{ acid} \times 0.698}{\text{Wt. of sample}}$$

TITRIMETRIC METHOD

Procedure.[8]—Prepare a solution of acetone containing 2% of water, and, another solution of 0.1% bromophenol blue in 95% alcohol. Mix 0.5 ml. of the indicator solution with 100 ml. of the acetone-water solution and then add 0.01 *N* hydrochloric acid to the first appearance of a permanent yellow color.

Place 40 g. of sample in a test tube of convenient size and add to this one ml. of water. Warm the mixture and shake it vigorously. Add 50 ml. of the neutralized acetone to the soap solution, warm and shake again and then allow the mixture to stand until the layers separate. The presence of soap is indicated by a green or blue color in the upper layer.

Titrate with 0.01 *N* hydrochloric acid to the reappearance of the yellow color. Continue the process of warming, shaking and titrating until the yellow color of the upper layer remains permanent.

$$\% \text{ Soap as sodium oleate} = \frac{\text{ml. } 0.01\ N \text{ acid} \times 0.304}{\text{Wt. of sample}}$$

This method is reported to be satisfactory for determining soap in oil within the range of 10–500 parts per million. The size of sample may be decreased for relatively high levels.

Marterana, Carl - Personal Communication

Witco Chemical

July 3, 1973

Tracor Jitco, Inc.
1300 East Gude Drive
Rockville, Md. 20851

Attention: Mr. Frank J. Letkiewicz, Chemist

Dear Sir:

In response to your request of June 14, 1973, addressed to our New York Office, we're submitting, herewith, the information we have available.

1. Specifications on Sodium Oleate Technical & Sodium Palmitate Technical & Sodium Oleate EA Food Grade

2. Stability in Containers
Generally speaking, soaps made from the saturated fatty acids (i.e. stearic, palmitic, etc.) have greater long term stability than those made from the unsaturated fatty acids (i.e. oleic). However, in a closed container, maintained at room temperature, the stability of the oleate is not a problem as it will keep at least a year. Exposure to air, higher temperatures and humidity will cause degradation of the product.

3. Usage and Use Levels
   a.) Sodium Oleate
   Thickener & emulsifier for aqueous systems at 1 - 5% levels.

   b.) Sodium Palmitate
   Similar uses to above. Additionally, it can act as a lubricant for powdered products.

cont

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Witco Chemical Corporation
Organics Division, 6200 W. 51st St., Chicago, Illinois 60638

Cable Address: Witcochem
Chicago 312-767-8771/Suburbs 312-458-0765

The amount we sell for food use is very limited, and we have little information on specific end application.

We hope that the information submitted is to your satisfaction.

Very truly yours,

WITCO CHEMICAL CORPORATION

Carlo Martorana

Carlo Martorana
Assistant Manager
Technical Service

CM/kp

encl: Specifications: Sodium Oleate Technical
Sodium Oleate Food Grade
Sodium Palmitate Technical

Witco Chemical

7/03/73

SPECIFICATIONS

SODIUM OLEATE TECHNICAL

&

SODIUM OLEATE EA FOOD GRADE

		Min.	Max.
Free Fatty Acid	-	--	2.5
Moisture	-	--	2.0
Ash (as Na_2CO_3)	-	17.0	18.0
Free Alkalinity	-	-- None --	
Fineness - 100 Mesh, %	-	50	--
20 Mesh, %	-	100	--
Color	-	Cream to light tan	

The specifications for the above grades are identical, however, the Sodium Oleate EA Food Grade is made from food grade oleic acid.

The technical grade is subject to 500# min. order quantity.

The food grade product is subject to 4000# min. order quantity and a lead time of approximately 2 weeks.

CM/kp

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Acta Med. Okayama 18, 247–259 (1964)

EFFECT OF SODIUM OLEATE ON THE METABOLISM AND SIZE OF RAT LIVER MITOCHONDRIA

I. UNCOUPLING, SHRINKAGE AND THEIR REVERSAL ACTION BY BOVINE SERUM ALBUMIN

Goki YAMAMOTO

Department of Biochemistry, Cancer Institute of Okayama University Medical School, Okayama, Japan (Director: Prof. M. Yamamoto)

Received for publication, may 8, 1964

Since the presence of uncoupler for oxidative phosphorylation as the control mechanism of cell metabolism was suggested by LARDY[1], several uncouplers have been isolated from various materials. These uncouplers have been detected including long-chain fatty acids, and the possible role of the long-chain fatty acids as the regulator of oxidation metabolism has been reported by several authors, e. g. in microsome by FEESSMAN and LARDY[2], in aged liver mitochondria by POLIS and SIMUCKLER[3], by PULLMAN and RACKER[4], in mitochrome by HULSMANN and co-workers[5,6], in insect mitochondria by WOJTCZAK and WOJTCZAK[7], in ascites tumor cells by SCHOLEFIELD and co-workers[8,9] and in hepatoma mitochondria by EMMELOT and BOS[10,11].

LEHNINGER and REMMERT[12] also extracted uncoupling factor (so-called U-factor) for oxidative phosphorylation from aged mitochondrial fragments, which causes the swelling of mitochondria. The activity of U-factor could be simulated by sodium oleate[12], and later WOJTCZAK and LEHNINGER[13] identified U-factor as long-chain fatty acids. The effect of fatty acid on rat liver mitochondria has been reported by many investigators[7,8,14–18]. Concerning the swelling and uncoupling of mitochondria the author[14] has demonstrated that the swelling action of mitochondria is stronger with unsaturated fatty acids than that with saturated ones, and that the extent of uncoupling activity of the fatty acids is parallel to that of the swelling action for mitochondria as a function of carbon chain length. There exists an interesting relationship between the uncoupling oxidative phosphorylation and the swelling. Besides this, the latent ATPase activity is also correlated to the uncoupling and swelling of mitochondria[7,14], and the uncoupling action of fatty acid for phosphorylation is protected[12,13,19–21] or reversed[1,4,15] by serum albumin.

For the purpose to reveal the action of sodium oleate on the mutural relationship between the function and structure of mitochondria, the changes of the respiration, oxidative phosphorylation, ATPase activity affected with oleate and

Witco Chemical

July 3, 1973

SPECIFICATIONS

SODIUM PALMITATE TECHNICAL

		Min.	Max.
Free Fatty Acid	-	0.2	1.3
Moisture	-	1.5	2.5
Ash (As Na_2CO_3)	-	17.0	17.5
Free Alkalinity	-	-- NONE --	
Fineness - 100 Mesh, %	-	40	--
Color	-	Equal to Standard	

Sodium Palmitate is a specialty item subject to 4000# minimum order quantity and two weeks lead time.

While we do not list a food grade Sodium Palmitate, it is a product which we can make, given sufficient potential. It, too, would be subject to the 4000# min. order quantity and needed lead time.

CM/kp

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For the purpose to reveal the action of sodium oleate on the mutural relationship between the function and structure of mitochondria, the changes of the respiration, oxidative phosphorylation, ATPase activity affected with oleate and

the reversal action of bovine serum albumin for uncoupling have been observed in connection with the swelling-shrinkage of rat liver mitochondria induced by oleate.

MATERIALS AND METHODS

The mitochondria were isolated from rat liver by a modified method of HOGEBOOM[22] as discribed by UTSUMI[23], a single exception being with and without ethylenediamine tetraacetate (EDTA), and suspended finally as stock mitochondria in a volume of 0.25M sucrose so that each milliliter represented the mitochondria obtained from 2g wet weight of the liver. Mitochondrial protein content was determined by the Kjeldahl technique[24].

The oxygen uptake of mitochondria was measured by the rotating platinum electrode, and the swelling-shrinkage was by the 90° light-scattering at 520mμ. Both measurements were carried out at 25° simultaneously with the use of the apparatus which was designed and constructed by UTSUMI[23]. Standard incubation mixture was consisted of 0.05M sucrose, 20mM KCl, 5mM K-phosphate, 10mM Tris-HCl buffer (pH 7.4), 100μM EDTA and with or without 1mM $MgCl_2$. Other experimental details of individual experiment are given in the legends to each figure.

Adenosine triphosphatase (ATPase) activity was tested at 25° for 10 minutes with gentle shaking in air environment. The incubation medium was composed of 0.1M sucrose, 20mM KCl, 10mM Tris-HCl buffer (pH 7.4), 40μM EDTA and 3mM ATP. The test in varying concentrations of sodium oleate was carried out in the presence or absence of bovine serum albumin (BSA) or Mg^{++}. BSA and $MgCl_2$ when added were 4mg and 1mM respectively, making the final volume 2ml. Reaction was started by the addition of 0.1ml of the stock mitochondrial suspension and stopped with 1 ml of 24 per cent perchloric acid. After centrifugation inorganic phosphate (Pi) was determined by the method of TAKAHASHI[25].

Relative fluorescence intensity of intramitochondrial pyridine nucleotides was measured by a fluorometer containing oxygen electrode in the sample cell constructed by the author[18]. Experimental detail is given in the legend to the figure.

ADP and ATP were obtained from Sigma Chemical Co., BSA from Armour Labolatories (fraction V), antimycin A from Kyowa Hakko Co. and 2, 4- dinitrophenol from Ishizu Pharmaceutical Co. Oligomycin was donated by Dr. UTSUMI. Sodium oleate was prepared with NaOH and oleic acid obtained from Tokyo Kasei Co. and the hydrogen ion concentration was adjusted to 7.4 with diluted HCl just before use. Other chemicals were of reagent grade and the distilled water to make up the solution was deionized.

RESULTS

ADP/O ratio and respiratory control index[27] of the intact mitochondria isolated from rat liver showed 1.9 and 4.0 respectively with Pi and succinate. The oxygen uptake of the mitochondria was increased slightly by the addition of sodium oleate (8 μM), and ADP/O ratio and respiratory control index lowered to 1.25 and 2.3 respectively. With the increased oxygen uptake by oleate, the mitochondria showed an increase of light-scattering (shrinkage) with a short lag. This shrinkage was enhanced by the addition of ADP, and after phosphorylating the added ADP to ATP, the light-scattering decreased (swelling) to the level before the addition of ADP. When oxygen was exhausted, the reversal of swelling (shrinkage)[28] of mitochondria was observed coming back to the initial level in the scattering intensity (Fig. 1).

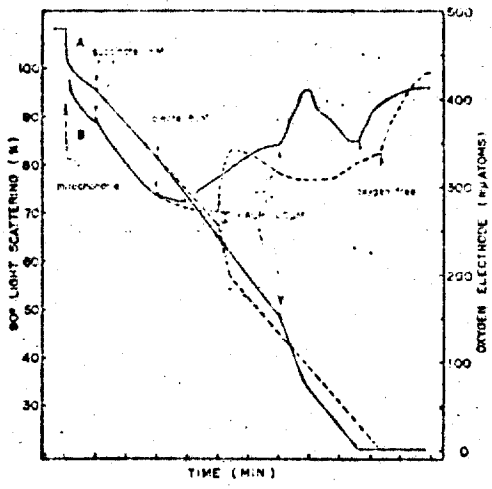


Fig. 1 Effect of sodium oleate on the respiration and swelling-shrinkage of rat liver mitochondria. The reaction mixture at 25° contained 50 mM sucrose, 20 mM KCl, 5 mM K-phosphate, 10 mM Tris buffer (pH 7.4) and 0.1 mM EDTA. Reaction was started by the addition of 0.1 ml of stock mitochondria (3.0 mg protein) isolated in 0.25 M sucrose without EDTA, the final volume, 2 ml. Other additions were 1 mM Na-succinate, 8 μM Na-oleate and 100 μM ADP. The time moves from left to right, and traces A and B indicate oxygen electrode current and 90° light-scattering changes respectively. The latter was measured at 520 mμ, and the initial scattering intensity of the mitochondria in the basic reaction mixture was taken as 100%. The decreases in scattering (swelling), scattering increases (shrinkage) and oxygen uptake were in the presence (——) and absence (- - - -) of oleate.

The degree and velocity of the shrinkage induced by oleate were enhanced proportionately to the concentration of oleate up to 0.16 mM per 3 mg mitochondrial protein, accompanying the proportional increment of the initial

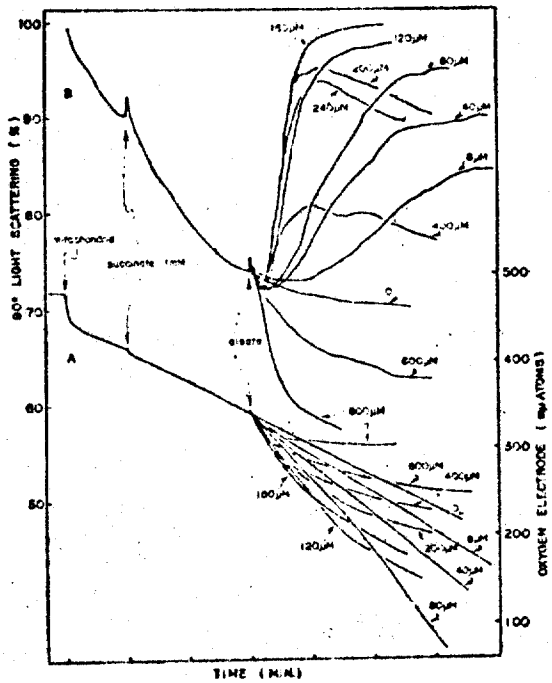
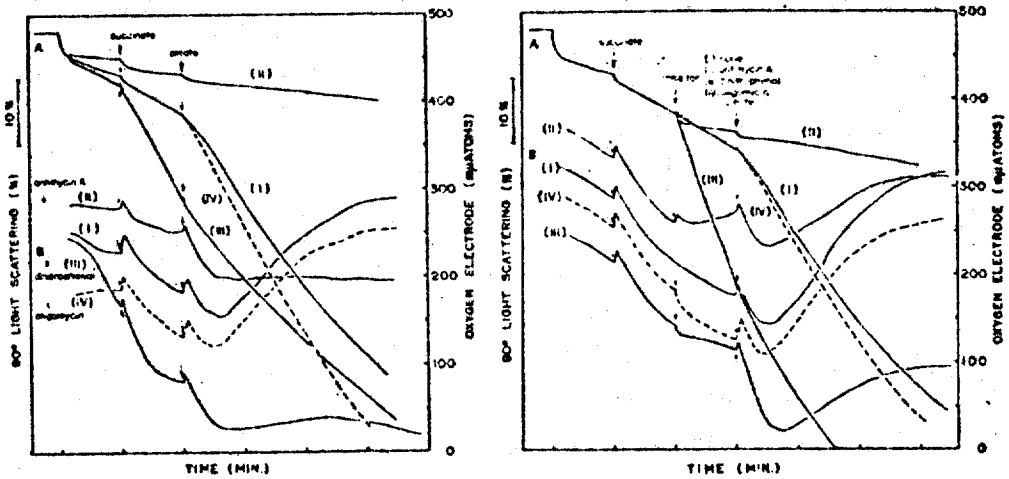


Fig. 2 Effect of various concentrations of sodium oleate on the swelling-shrinkage changes and respiration of rat liver mitochondria. Traces A and B refer to oxygram and light-scattering. The concentrations of added substances are in Fig. and the reacting conditions are as in Fig. 1.

velocity of respiratory release (Fig. 2). From the data of this experiment, it was calculated that about 0.1 μ mole of oleate was required to cause the greatest shrinkage of 1 mg protein mitochondria. In a higher concentration of oleate more than 0.1 μ mole per mg mitochondrial protein the degree of shrinkage was lowered proportionately to the increment of oleate concentration, though the initial velocity of the shrinkage was the same as observed in the case of the maximum shrinkage. Namely, the shrinkage ceased at a certain period of incubation after the addition of oleate accompanied by the fall-off of oxygen uptake. Oleate in the concentration of more than 0.2 μ moles per mg protein induced the prompt swelling of mitochondria without shrinkage, accompanied by the inhibition of the respiration.

The swelling-shrinkage change and the extent of oxygen uptake of mitochondria with oleate were affected by respiratory inhibitor, uncoupler, and inhibitor of phosphorylating respiration (Figs. 3 and 4). The period of the addition, before and after the incubation of mitochondria with Pi and succinate, affected the action of these inhibitors. On incubation of mitochondria with antimycin A,



Figs. 3 and 4 Effect of inhibitors on the oleate-induced shrinkage and respiratory release of rat liver mitochondria. The reaction mixture at 25° contained 0.1 M sucrose, 0.02 M KCl, 5 mM K-phosphate, 10 mM Tris buffer (pH 7.4), 0.1 mM EDTA and 1 mM $MgCl_2$. Reaction was started by the addition of mitochondria (2.4 mg protein) isolated in 0.25 M sucrose with 40 μM EDTA. Total volume was 2 ml. Other additions were 3 mM Na-succinate, 0.12 mM Na-oleate, 4 μg antimycin A, 0.05 mM 2, 4- dinitrophenol and 4 μg oligomycin. Traces A and B refer to oxygram and light-scattering. Fig. 3 shows the effect of inhibitors added before the incubation of mitochondria with succinate and Fig. 4 shows the one added after the incubation. Curves (I), (II), (III) and (IV) indicate those in the absence of inhibitor, in the presence of antimycin A, 2, 4- dinitrophenol and oligomycin, respectively.

2,4-dinitrophenol, or oligomycin before the addition of succinate, the mitochondrial shrinkage with oleate was inhibited by antimycin A or by dinitrophenol but not by oligomycin, and the oxygen uptake of mitochondria treated with oleate was inhibited with antimycin A or with 2, 4- dinitrophenol but increased by oligomycin (Fig. 3). These inhibitor patterns were changed by the addition of inhibitors after the pre-incubation of mitochondria with Pi and succinate (Fig. 4). The inhibitor patterns of oxygen uptake was same as in the case of preincubation with these inhibitors but the shrinkage with oleate was observed even in the presence of antimycin A or 2, 4- dinitrophenol.

By the addition of BSA, ADP/O ratio and respiratory control index of the mitochondria were not altered, and the mitochondrial structure was also not affected by BSA, as the pattern of swelling-shrinkage induced by ADP was not altered (Fig. 5) Thus, BSA has no effect on oxidative phosphorylation linked with succinate in fresh liver mitochondria under the present experimental conditions.

After addition of a small amount of oleate (0.05 mM) to mitochondria which induces the increased oxygen uptake and the marked shrinkage (Fig. 6 trace I), a slight increase of respiration and shrinkage were brought about by the

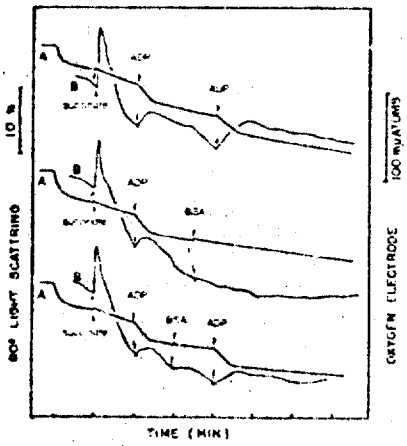


Fig. 5 Effect of BSA on the phosphorylation of rat liver mitochondria. Preparation of mitochondria and the reaction mixture are the same as in Fig. 1. Mitochondrial volume used in this experiment was 1.6 mg protein and other additions were 10mM Na-succinate, 40μM ADP and 2mg BSA. Traces A and B refer to oxygram and light-scattering.

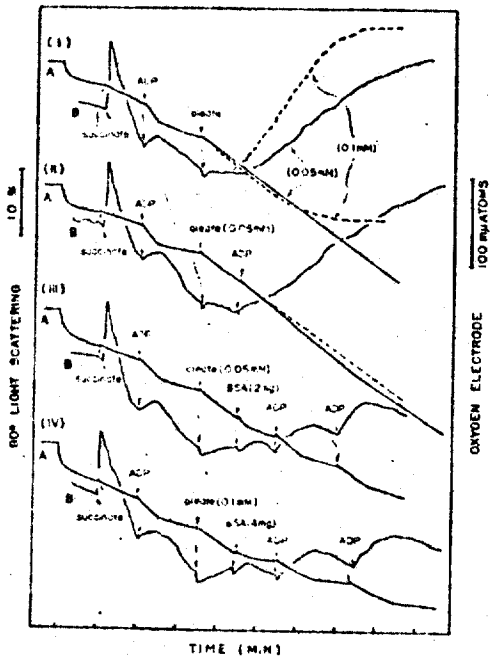


Fig. 6 Reversal by BSA from the uncoupled oxidative phosphorylation and shrinkage of rat liver mitochondria induced by sodium oleate. Preparation of mitochondria and reaction mixture are as shown in Fig. 1 except 1.6 mg protein mitochondria. The concentrations of oleate and BSA are given in Fig. and of Na-succinate and ADP are 10 mM and 40μM, respectively. Traces A and B refer to oxygram and light-scattering.

addition of ADP showing the decrement of the ADP/O ratio and respiratory control index (trace II). But by the addition of 2 mg BSA after the treatment with oleate, the increased oxygen uptake by oleate was decreased and the swelling induced, and then the ADP/O ratio and respiratory control index were observed steadily by the addition of ADP (trace III). On the other hand, when a higher concentration of oleate (0.1 mM) was added to mitochondria, the oxygen uptake was increased but was followed by fall-off, and the increased scattering was greater in extent than that of oleate at a low concentration (trace I). In this instance, it required about 4 mg BSA to reverse the respiratory release and the shrinkage (trace IV). This reversal of uncoupling was not induced with 2 mg BSA, however there could be observed the increased respiration without fall-

off and the decreased shrinkage. Moreover, in the presence of BSA in a sufficient amount before the treatment with oleate, the mitochondrial respiration and swelling-shrinkage were not affected by added oleate.

The ATPase activity was increased proportionately to the increasing of concentration of oleate up to about 0.1 μ mole per mg protein of mitochondria and decreased gradually by that of oleate higher than 0.1 μ mole (Fig. 7). The con-

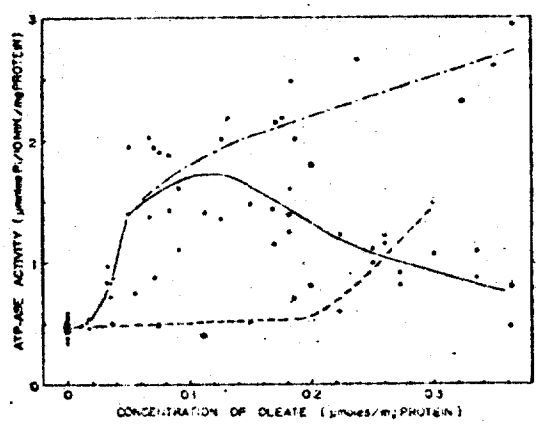


Fig. 7 The ATPase activity of rat liver mitochondria induced by sodium oleate in varying concentrations. Experimental conditions are as described in the text. ⊖, ——, oleate; ⊙, ----, oleate + 4mg BSA; ⊙, ---·---, oleate + 1mM $MgCl_2$

centration of oleate to induce the maximum activity of ATPase was in the same range as in the case of the maximum mitochondrial shrinkage. This oleate-induced latent ATPase activity was protected by BSA, but this protective action of BSA (4mg) was abolished progressively with the increasing of oleate ranging 0.2—0.4 μ moles per mg protein of mitochondria. The concentration of BSA to abolish the oleate-induced latent ATPase activity coincided with that of BSA to protect the uncoupling and shrinkage action of oleate. On the other hand, the ATPase activity induced by oleate was stimulated by the presence of Mg^{++}, and this stimulated activity was increased gradually by the increasing of oleate concentration, agreeing with the reports of other investigators[2,15,17].

Relative fluorescence intensity (reduced pyridine nucleotides) of rat liver mitochondria was reduced by added Pi and increased by succinate (Fig. 8). The addition of ADP induced the increase of the intensity accompanied with the increase of respiration with respect to the respiratory control. Then after phosphorylation of ADP to ATP the fluorescence intensity increased to the level of steady state, as described by CHANCE and HOLLUNGER[29]. The decrease of fluorescence intensity and increase of respiration were induced by the addition of oleate

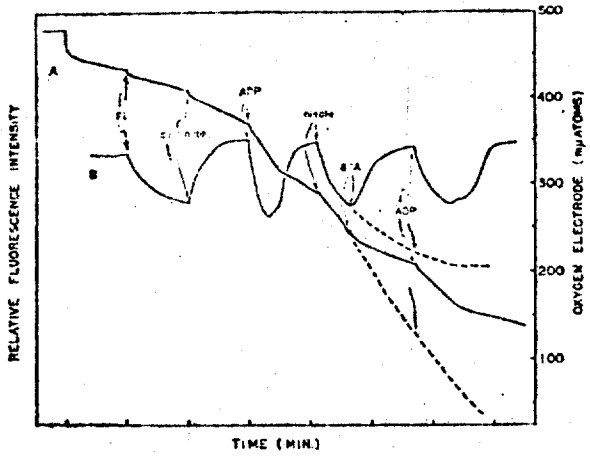


Fig. 8 Changes of relative fluorescence and respiration of rat liver mitochondria. Both measurements were carried out simultaneously at 25° in final 2 ml of a medium consisted of 0.1 M sucrose, 0.02 M KCl, 50 μM EDTA, 1 mM $MgCl_2$ and 10 mM Tris buffer (pH 7.4). The reaction was started by the addition of 2.1 mg protein mitochondria and other additions were 3 mM K-phosphate (pH 7.4), 3 mM Na-succinate, 100 μM ADP, 0.16 mM Na-oleate and 4 mg BSA. Trace A indicates oxygen uptake and trace B relative fluorescence. The latter trace shows the state of oxidation-reduction of intramitochondrial pyridine nucleotides (arbitrary unit). The downward deflection refers to the oxidation and upward to the reduction. Broken line denotes the changes in the absence of BSA.

at the same time. On additing BSA, the increased respiration induced by oleate was reversed and the fluorescence intensity decreased to the level of steady state, and then the respiratory control with ADP accompanied by the oxidation-reduction of fluorescence could be seen again.

DISCUSSION

It has been clarified that the mitochondria manifest structural changes, as measured by 90° light-scattering in their membrane coupled with oxidative phosphorylation[23,30-34]. The extent of swelling with oleate is changed not only by the concentration of oleate[13] but also by varying incubation mixture[18,33]. Oleate induces high amplitude swelling[34] in KCl-Tris buffer solution but low amplitude swelling[34] in sucrose-Tris buffer solution. This low amplitude swelling induced by oleate is worthy of attention with respect to the endogenous regulator of oxidation metabolism, because the activity of endogenous uncoupler can be produced by oleate[12]. In fact, this uncoupling oxidative phosphorylation with oleate is closely correlated with the swelling-shrinkage of mitochondria, as can be observed from the fact that the extent of mitochondrial shrinkage induced by

oleate parallels the extent of respiratory release linked to succinate. Furthermore, there has been observed quantitative relationship between the amount of mitochondria and that of oleate manifesting the consistent activity for uncoupling and shrinkage.

The shrinkage and uncoupling of mitochondria induced by oleate are protected and readily reversed by serum albumin. The protective action of albumin has been observed in skeletal muscle mitochondria[21,28], in insect mitochondria[7], in fresh isolated tumor mitochondria[6], and in aged mitochondria[4,29]. As to this protective action, it may be explained that serum albumin binds to long-chain fatty acids, particularly to oleic acid, so tightly that the two could not be separated electrophoretically[37,38]. Serum albumin, on the other hand, strongly inhibits the swelling of mitochondria caused by oleate[12,13,15]. However, little is known[15,20] about the reversibility of uncoupling and swelling by serum albumin after mitochondria have come in contact with oleate. The present investigation shows that a complete reversal action could be demonstrated by the addition of a sufficient amount of BSA on the uncoupling, on the structural change, and on the oxidation of pyridine nucleotides induced by oleate. Furthermore, oleate-induced latent ATPase activity was also abolished by addition of BSA. However, the mitochondrial respiration accompanied by the swelling in high amplitude by a large amount of oleate was not reversed completely by addition of a sufficient amount of BSA. Therefore, the action of oleate in a low concentration may be distinguished from the break-down of the mitochondrial structure and may have a physiological meaning such as regulation of energy metabolism. In this instance, if the control of the cell metabolism by the presence of uncoupler, fatty acids, is possible[1], the relationship between the uncoupling of such amount of oleate and the ready reversal of this uncoupling by BSA, regardless of how BSA may protect reversibility under any circumstance, may be considered as the action as regulator of cell metabolism.

Many investigators have been studying the action of oleate on mitochondria but the mechanism remains obscure. The increment of mitochondrial respiration induced by oleate may be due to the following three possibilities: First one is the oxidation of fatty acid by mitochondria. This possibility was proposed by WOJTCZAK *et al.*[13,18] from the study of C^{14}-oleic acid oxidation, and several investigators described that oxidation of fatty acids is increased by the formation of fatty albuminate[38-40] without uncoupling of oxidative phosphorylation. Furthermore, as described in this paper, the reduction of pyridine nucleotides by BSA after treatment of oleate would be proposed the first posibility. The second is the oleate-induced ATPase activity. ADP increased by ATPase induces the respiratory control, increasing respiration. In fact, the ATPase activity is induced by oleate as described in the present report but the produced ADP by the oleate-

induced ATPase can not bring about the increase of respiration, because oligomycin did not inhibit this increased respiration. The third is the uncoupling oxidative phosphorylation.

Mechanism of the increased respiration by true uncouplers such as dinitrophenol and oleic acid has been accounted for by the discharge of high energy intermediate, initial step of oxidative phosphorylation[8,41,42]. On the other hand, the mechanism of mitochondrial shrinkage by oleate is accounted for by the decrement of high energy intermediate of oxidation as observed in the shrinkage induced by ADP, dinitrophenol, antimycin A or anaerobiosis[23,31,33]. In the present data the shrinkage with oleate is inhibited slightly by addition of antimycin A or 2, 4-dinitrophenol after prior incubation with Pi and succinate, since it would already slightly decrease the high energy intermediate by these inhibitors, but the inhibition is accerelated by preincubation with antimycin A or 2, 4-dinitrophenol before additions of Pi and succinate. In contrast to these inhibitors, oligomycin does not inhibit the oleate-induced respiration and shrinkage. Therefore, it can be considered that the energized intermediate acted on by oleate exists in an initial step of oxidative phosphorylation processes. In this case, oleate at a higher concentration would bind to the intermediate as demonstrated by HEMKER[43] for the explanation of the uncoupling mechanism of dinitrophenol. Thus, the fall-off of increased respiration accompanied by the shrinkage ceased by oleate would be explained by the inhibition of the regeneration of intermediate in phosphorylation sequence.

With respect to the idea related to the intermediate, furthermore, it is supported by the evidence of ATPase activity: both oleate-induced ATPase and shrinkage are increased in its activity and degree proportional to the concentration of oleate, showing that the degree of shrinkage is dependent upon the quantity of intermediate. On the other hand, the decreasing ATPase activity by increasing concentration of oleate is due to disruption of the mitochondrial structure, because the Mg^{++}-stimulated ATPase activity is induced proportional to the decrement of oleate-induced ATPase activity and also to the increment of the degree of swelling (high amplitude).

The high amplitude swelling is identified from the low amplitude swelling induced by a low concentration of oleate with respect to the control mechanism of energy metabolism in the cell, and the physical and physiological actions of oleate differ by its concentration. The extent of shrinkage induced by oleate is much greater than that by other reagents. This phenomenon cannot be explained satisfactorily simply by the mechanism of uncoupling action regarding the high energized intermediate. For the action of oleate it must await further experimental examinations, but it is considered that the interaction of the synthesis of phospholipids from oleic acid is accompanied with the ATP-induced contrac-

tion of swollen mitochondria[18].

SUMMARY

Effects of sodium oleate and bovine serum albumin (BSA) on rat liver mitochondrial function and structure were studied by measuring oxygen uptake, 90° light-scattering, adenosine triphosphatase activity and pyridine nucleotides fluorescence.

1. The low concentration of oleate induced the uncoupling of oxidative phosphorylation and the scattering change of mitochondria. This action of oleate differed from that of oleate at a high concentration which induces the high amplitude swelling with respect to its physiological and biochemical properties. The degrees of reversal swelling (shrinkage) and of oxygen uptake induced by oleate in the presence of Pi and succinate were altered proportionately to the concentration of oleate, and the concentration of oleate to the shrinkage coincided with that of the maximal respiratory release.

2. Antimycin A or 2, 4-dinitrophenol prevented the oleate-induced mitochondrial shrinkage, but the treatment of these agents after prior incubation with Pi and succinate allowed the shrinkage, though the degree was small in its extent compared with that in the absence of inhibitors. On the other hand, oligomycin did not affect the shrinkage with oleate.

3. BSA protected the mitochondrial phosphorylation from the uncoupling action of oleate without showing any effect of its own. A complete reversal could readily be demonstrated by a sufficient amount of BSA from the uncoupling, structural changes, and oxidation of intramitochondrial pyridine nucleotides induced by oleate in a low concentration.

4. The oleate-stimulated latent ATPase activity was proportional to the oleate-induced shrinkage of mitochondria with respect to the concentration of oleate. The latent ATPase was abolished also by the addition of a sufficient amount of BSA.

5. The action of oleate on the phosphorylation sequence of mitochondria was discussed on the basis of the present findings.

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