MONOGRAPH ON TALLOW AND STEARIC ACID #59

Monograph on Tallow & Stearic Acid

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P29

MONOGRAPH

ON

TALLOW

AND

STEARIC ACID

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TALLOW

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TALLOW

Summary

Tallow is fat rendered from the tissue of bovine cattle and sheep and is therefore, in an unrefined state, a part of the standard human diet. In the form of glycerides in both plant and animal products, stearic acid is also regularly consumed by man. In their natural forms these compounds are apparently harmless. However, these substances are currently being used as food additives in refined forms and, therefore, it is of interest to review their biochemistry and toxicology. Since the primary constituents of tallow are glycerides, it is suggested that the monograph on glycerine and glycerides be reviewed in conjunction with this monograph.

Considerable information is available on the biochemical aspects of tallow and stearic acid.

In numerous studies using chickens as test subjects, the absorbability of tallow has been calculated as 53-82%, varying with age of the chickens and the level of tallow in the diet (203, 635, 838, 492). Metabolizable energy (ME) values of 6.02-7.69 kcal/g were determined for tallow while stearic acid was found to have a negative ME value (635, 601, 697, 629). Hoagland and Snider (305) found that in the rat stearic acid is very poorly absorbed and exhibits a digestibility coefficient of 9.4-21.0%. Bayley and Lewis (58) and Carlson and Bayley (107) determined the digestibility of tallow and stearic acid by pigs to be 56-85% and 60%, respectively. Raven (624) reported the digestibility to be approximately 90% for calves.

According to Kayden et al. analysis of intestinal lumen contents of man after fat feeding has shown a separation of glycerides into two phases: an oil phase which contains mainly diglycerides and triglycerides and some fatty acid, and a micellar phase which is a combination of bile salts, free fatty acids, and monoglycerides. From the micellar solutions monoglycerides and free fatty acids are absorbed through the intestinal wall, leaving the bile salts within the lumen to form additional micelles. The fate of the free fatty acids within the mucosal cell is determined by their chain length and saturation; those of less than 12 carbons are preferentially absorbed by rats directly into the portal blood, whereas longer chain fatty acids are incorporated mainly into triglycerides, and appear in lymph chylomicrons. Monoglycerides within epithelial cells may be esterified to di- and triglycerides or cleaved to glycerol and free fatty acids. The 2-monoglycerides are esterified especially rapidly to triglycerides, but esterification of 1-monoglycerides lags and these are more likely to undergo hydrolysis particularly if the fatty acids are of short chain length (399).

Fatty acids play an important role as an energy source. Their oxidation is prominent in higher animals which can store large amounts of neutral fat as a fuel reserve. It is estimated that fatty acid oxidation provides at least half of the oxidation energy in the liver, kidneys, heart muscle and resting skeletal muscle. 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 triacylglycols but very small amounts of free fatty acids. The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consists largely of triacylglycerol. Before the fatty acids can undergo activation and oxidation, the triacylglycerols first must undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerols. Free fatty acids are therefore derived either from the blood stream, in which they are transported bound to serum albumin, or prior hydrolyses of intracellular lipids. They are first activated by esterification with CoA to form acyl CoA esters of the outer mitochondrial membrane. Then the fatty acyl group from CoA is carried across the inner membrane by the carrier molecule carnitine. Finally the fatty acyl group is transferred from carnitine to intramitochondrial CoA: through several enzymatic reactions. A two-carbon fragment of the fatty acid is removed as acetyl CoA and two pairs of hydrogen atoms are given to specific acceptors. A long chain fatty acid will go through several spirals. Thus the 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules of acetyl CoA and 14 pairs of hydrogen atoms. The acetyl CoA formed as a product of the fatty acid oxidation will then enter the tricarboxylic acid cycle, which is the final common pathway of oxidative catabolism of all fuel molecules in aerobic cells (452).

Lin et al. (465) reported that the consumption of stearic acid by chickens results in increased hepatic cholesterol synthesis. According to Janssen (365), stearic acid ingestion results in increased plasma cholesterol in rats. Dietary studies by Hegsted et al. (280) using 21 middle-aged male mental patients indicate that stearic acid plays a significant role in elevating serum cholesterol. However, Karvinen et al. (387) reported that oral administration of 30 g of stearic acid per day to 6 young healthy adult humans did not significantly influence the absorption of dietary cholesterol and Grande et al. (241) concluded on the basis of his studies that stearic acid has no effect on the serum cholesterol concentration in man. Renaud and his coworkers (632, 629, 630) reported that stearic acid and tallow appear to be thrombogenic when included in the hyperlipemic diet of rats and that stearic acid also exhibits a strong atherogenic effect.

Several studies have been reported on the toxicity and carcinogenicity of stearic acid. The only studies reported for tallow, however, have been short-term feeding studies with chicks and those were more concerned with nutrition than toxicology.

On the basis of studies in which mice were injected subcutaneously with stearic acid, Van Duuren et al. (796), Swern et al. (738), and Hieger (288) all concluded that stearic acid is not carcinogenic. Herting and Crain (286) observed a foreign-body type reaction in fat taken from rats after maintainence for 8 weeks on a diet containing 50% stearic acid.

Oro and Wretlind (571) determined the LD_{50} of stearic acid to be 23± 0.7 mg/kg when injected into mice i.v. Doses near this level produced immediate convulsions and cessation of respiration within 1-2 minutes (571).

The feeding studies reported in which chickens were used were concerned primarily with the possible use of tallow and stearic acid in commercial chicken feeds. Administration of rations containing up to 5% stearic acid or 20% tallow were found to produce no deleterious effects (733, 628, 458, 61, 687, 834). In most cases weight gain and feed efficiency were improved by the addition of these substances (733, 628, 458, 61, 687, 834).

To study the toxicity of stearic acid, Price and Beutner (617) maintained young white rats on a diet containing 50% stearic acid. The male rats died after an average of 8.2 days, the females after 10.2 days (617). Shortly before death the rats showed cardiac irregularities and in some cases there was spasm and paralysis of the extremities (617). In further experiments in which rats were given 3/10 the dose of stearic acid, death was considerably delayed (617). The addition of as little as 5% glyceride oil was found to markedly diminish the toxicity of stearic acid (617). In a short-term feeding study by Deichmann et al. (159), 10 Sprague-Dawley rats were fed 3000 ppm of stearic acid in a basal diet for a period of 209 days. The test rats exhibited anorexia, severe pulmonary infection, and high mortality (159).

TALLOW

Chemical Information

I. Nomenclature

A. Common Name

1. Tallow
2. Oleo Stock
3. Beef Tallow

B. Chemical Names

None

C. Trade Names

None

D. Chemical Abstracts Registry Number

977002-72-4

II. Empirical Formula

Not Applicable

III. Structural Formula

Not Applicable

IV. Molecular Weight

Not Applicable

V. Specifications

Not Available

VI. Description

A. General Characteristics

In North America the term tallow designates the fat from the fatty tissue of bovine cattle and sheep only. It contains, as glycerides, oleic (37-43%), palmitic (24-32%), stearic (20-25%), myristic (3-6%) and linoleic (2-3%) acids. Minor constituents are cholesterol, arachidonic, elaidic, and vaccenic acids.

An analysis of Grade B animal tallow yielded the following results (697):

Gross energy Cal./gm.	9.32
Protein %	0.1
Ether extract %	94.4
Moisture %	0.87
Free fatty acids %	4.8
Iodine value	56.4
Titer °C.	40.8
Unsaponifiable %	0.96
Saponification value	198.8
Fatty acid composition %	
C_8	Trace
C_{10}	0.05
C_{12}	0.08
C_{14}	2.50
C_{14} (Double bond)	0.46
C_{15}	0.18
C_{16}	26.9
C_{16} (Double bond)	3.3
C_{17}	0.35
C_{18}	17.4
C_{18} (Double bond)	41.5
C_{18} (2 Double bonds)	7.5
C_{18} (3 Double bonds)	0.31
C_{20}	—
C_{22}	

B. Physical Properties

The titer of tallow ranges from 40 to 46 degrees.

C. Stability

No Information Available

VII. Analytical Methods

Bartlett and Mahon developed an analytical technique whereby an unknown fat or oil may be identified at least within a group by differential infrared spectroscopy with olive oil as reference and positive identification made by the spectrum of the unknown with an oil of the group as reference (51).

VIII. Occurrence

A. Plants

None

B. Animals

Tallow is the fat from the fatty tissue of bovine cattle and sheep.

C. Synthetics

None

D. Natural Inorganic Sources

None

STEARIC ACID

Chemical Information

I. Nomenclature

A. Common Names

1. Stearic Acid

B. Chemical Names

1. n-Octadecanoic Acid

C. Trade Names

None

D. Chemical Abstracts Registry Number

000057-11-4

II. Empirical Formula

$C_{18}H_{36}O_2$

III. Structural Formula

$CH_3(CH_2)_{16}COOH$

IV. Molecular Weight

284.47

V. Specifications

U.S.P. stearic acid consists chiefly of a mixture of stearic and palmitic acids. It is in the form of white or slightly yellow, crystalline masses, or a white to slightly yellow powder; slight tallow-like odor. Does not congeal below 54 degrees.

Typical specifications for commercial triple-pressed stearic acid are: Titer: 54.5-55.0 degrees; color 5 1/4" Lovibond red: 0.1-0.5; 5 1/4" Lovibond yellow: 1.0-2.0; saponification value 207-210; acid value 206-209; iodine value 3.5-4.5. Typical specifications for commercial double-pressed stearic acid are: Titer: 53.9-54.3 degrees; color 5 1/4" Lovibond red: 0.5-1.0; 5 1/4" Lovibond yellow: 2.0-3.0; saponification value 207-210; acid value 206-209; iodine value 5.5-7.0.

VI. Description

A. General Characteristics

Pure stearic acid exists in the form of white leaflets.

B. Physical Properties

Stearic acid has the following characteristics: d^{70} 0.847; mp 69-70 degrees; bp 383 degrees; n_D^{80} 1.4299. It slowly volatilizes at 90-100 degrees. Stearic acid is practically insoluble in water. One gram dissolves in 21 ml alcohol, 5 ml benzene, 2 ml chloroform, 26 ml acetone, 6 ml carbon tetrachloride, 3.4 ml carbon disulfide; also soluble in amyl acetate and in toluene.

C. Stability

Store in well-closed containers.

VII. Analytical Methods

Kato and Yamaura developed a rapid gas chromatographic method for the determination of fatty acid compositions of soybean oil and castor oil. In their method, the first part of a gas chromatography column is packed with Chromosorb W AW (60-80 mesh) impregnated with NaOH or KOH, and the remainder is packed with 25% diethylene glycol succinate on Chromosorb W AW (60-80 mesh) for soybean oil and other oils and fats and with 3% silicon SE-30 on Chromosorb W AW (100-120 mesh) for castor oil. Soybean oil is injected in 1:1 benzene-MeOH solution, and castor oil in MeOH solution. The injection port and column temperature was 210 degrees for castor oil and 200 degrees for other oils and fats. Transesterification of the oils to the Me esters occurs rapidly in the first part of the reactor and the esters are consequently analyzed (390).

VIII. Occurrence

A. Plants

Stearic acid occurs as a glyceride in some vegetable oils.

B. Animals

Stearic acid occurs as a glyceride in tallow (20-25%) and other animal fats and oils.

C. Synthetic

Stearic acid is prepared synthetically by hydrogenation of cotton seed and other vegetable oils.

D. Natural Inorganic Sources

None

Biological Data

I. Acute Toxicity

Substance	Animal	Sex & No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Stearic Acid	Mice	60	i.v.	23	LD_{50}	571

To determine the acute toxicity of stearic acid, Oro and Wretlind injected mice (6 groups of 10; 13-34 g) intravenously with an 0.2% emulsion. An LD_{50} of 23± 0.7 mg/kg was determined. When doses near this level were injected the animals immediately had convulsions and collapsed on their sides. Respiration ceased within 1-2 minutes, sometimes after hyperpnoea (571).

II. Short-term Studies

Chickens

Groups of 13-25 day-old chicks were maintained for 4 weeks by Sunde on a chick starting diet containing 5% prime tallow, No. 1 tallow, or stearic acid. No deleterious effects resulted from the consumption of any of these diets. Feed utilization was improved by tallow but not by stearic acid (733).

Groups of 11 male and 11 female one-week-old Indian River Cross chicks were maintained by Reiser et al. on rations containing 7, 11 or 15% tallow for a period of 9 weeks. During the first 5 weeks, at least, tallow increased the feed efficiency proportionally to the increased caloric value of the rations. However, tallow had no influence on rate of growth (628).

Leveille and Fisher conducted a study in which groups of 12 Single Comb White Leghorn hens were fed rations containing no fat or 10% tallow, each at levels of 15 and 25% protein, from 12 weeks of age. No significant differences were observed in days to initial egg, egg size, egg production, or feed efficiency. Hens receiving tallow were heavier than the controls and hens on low protein diets exhibited higher body weights than the birds on the high protein level. The animals receiving tallow also exhibited elevated plasma cholesterol (458).

In a study by Beilharz and McDonald, newly hatched White Leghorn X Australorp cross bred cockerels, in groups of 25, were fed a diet containing 5% tallow for a period of 12 weeks. The tallow did not affect growth rate but it improved feed efficiency (61).

For a period of 8 weeks, Sell and Hodgson maintained 3 groups of 15-day-old cockerels on a basal diet supplemented with 0, 4, or 8% animal tallow. At the higher level, tallow consumption significantly

increased weight gain and feed efficiency while at the lower level no effect was observed (687).

For a period of 4 weeks, Yacowitz et al. maintained 60 male White Plymouth Rock chicks on a 24.6% protein corn-soy basal ration supplemented with 20% fat, using either edible grade beef tallow or U.S.P. cocoa butter. During the test period the tallow-fed chicks averaged 576 g gain while the cocoa butter-fed chicks gained only 351 g. Serum lipid analysis at termination of the test period showed the following for tallow and cocoa butter, respectively (mean in mg/100 ml ± S.D.): total lipids, 586 ± 27, 388 ± 13; neutral lipids, 378 ± 38, 183 ± 29; phospholipids, 210 ± 24, 202 ± 43; total cholesterol, 153 ± 3, 102 ± 3; triglycerides, 123 ± 14, 84 ± 12. The mean oleic acid concentration of the serum fatty acids was somewhat higher with tallow than with cocoa butter (834).

Rats

To study the toxicity of stearic acid, Price and Beutner maintained young white rats on a diet of which one half of the total food was stearic acid and the remainder was casein, glucose, salt mixture, cellulose and vitamin mixture. Water was provided ad libitum. The male rats died after an average of 8.2 days, the females after 10.2 days. Shortly before death the rats showed cardic irregularities and in some cases there was spasm and paralysis of the extremities. In further experiments in which rats were given 3/10 the dose of stearic acid death was considerably delayed. Furthermore, the addition of as little as 5% glyceride oil, such as corn oil, was found to markedly diminish the toxicity of stearic acid (617).

In a short-term feeding study by Deichmann et al., two groups of 10 Sprague-Dawley rats (5 males and 5 females per group) were fed 3000 ppm of either octadecylamine or stearic acid in a basic diet for a period of 209 days. Records were kept of food consumption and body weight. At termination of the experiment gross and microscopic pathological examinations were made on tissues (brain, viscera, gonads, skeletal muscle, bone marrow, heart, lungs, liver, spleen, kidneys, gastroenteric tract (three levels), pancreas, and lymph nodes) taken from all animals. Both compounds produced anorexia and high mortality (see Table 1). The rats fed stearic acid showed no significant pathological lesions, but did exhibit severe pulmonary infection (159).

TABLE 1.—Effect of Feeding to Rats 3000 Ppm of Octadecylamine and 3000 Ppm of Stearic Acid for a Period of 209 Days

Compound	Sex	Mortality Ratio	Average Survival Time, Days	Average Weight Change, Gm	Average Diet Consumption per Day, Gm
Octadecylamine	Males	4/5	67	−31	9.8
	Females	1/5	149	+39	11.5
Stearic acid	Males	5/5	107	+12	15.0
	Females	3/5	127	+58	13.5

III. Long-term Studies

None

IV. Special Studies

Carcinogenesis

Van Duuren et al. reported the results of a series of bioassays for the carcinogenic activity of stearic acid and some related compounds. These compounds, with tricaprylin as vehicle, were injected s.c. into mice once weekly for 26 weeks in the doses shown in Table 1. On the basis of the results shown in Table 1, the authors concluded that stearic acid is not carcinogenic under the given conditions, while the other compounds studied exhibit low-level carcinogenic activity (796).

Table 1

Induction of sarcomas at injection site by s.c. injection in mice; results at 21 months

Compound	Laboratory	Dose (mg/0.1 ml tricaprylin) once weekly[a]	Total dose (mg)	No. of mice at start	No. of mice alive at 6 mo[b]	Sarcomas at injection site	Other tumors
Stearic acid	A[c]	0.5	13	15	14	0	1
		0.05	1.3	15	15	0	3
	B	0.5	13	16	14	0	0
		0.05	1.3	16	13	0	0
Methyl stearate	A	5.0	130	15	15	2	1
		0.5	13	15	15	0	3
	B	5.0	130	16	16	2 (15)[d]	2
		0.5	13	16	16	0	0
γ-Stearolactone	A	1.0	26	15	14	0	0
		0.05	1.3	15	14	1	1
	B	1.0	26	16	16	1 (15)	3
		0.05	1.3	16	16	1 (18)	2
p-Nitroperbenzoic acid	A	0.05	1.3	15	15	2	0
		1.0	26	16	7	0	0
	B	0.05	1.3	16	16	1 (15)	4
Glycidyl stearate	A	0.1	2.6	15	14	1	3
		0.05	1.3	15	14	1	5
	B	0.1	2.6	16	16	1 (16)	4
		0.05	1.3	16	16	1 (16)	1
Tricaprylin	A	0.1 ml		15	15	0	0
	B	0.1 ml		16	16	0	3
No treatment	A			15	15	0	0
	B			32[e]	23	0	3

[a] Injected s.c., once weekly, for 26 weeks in inguinal area.
[b] From beginning of experiments. Median survival ranged from 15 to 21 months in test and control groups except for *p*-nitroperbenzoic acid the higher dose (Group B only) where the median survival time was 5 months.
[c] A, New York University; B, Fels Research Institute.
[d] Nos. in parentheses, month when found.
[e] Some mice sacrificed for long-term observations to 6 months.

Table 2 presents the details and results of a series of tests by Swern et al. in which stearic acid was tested for carcinogenicity by s.c. injection into mice. Although one group of 10 mice developed 4 sarcomas, the authors concluded that stearic acid showed no carcinogenic activity under the conditions of the tests (738).

Table 2

Detailed data on tests for carcinogenic activity

No.	Compound	Year	Dose/injection (mg)	Sch. dule (injections No./wk/total injections)	Total dose (mg compound/ml tricaprylin)	Mouse strain	Mice alive at month 0	6	9	12	15	18	Tumors by month of appearance: Subcutaneous sarcomas	Pulmonary tumor	Other tumors
	Controls														
	Tricaprylin	1964		2/104	0/10.4	S[a]	40	38	32	28	23	12		23	B(12), S(17, 19); C(17)
	Tricaprylin	1965		3/12	0/1.2	S	15	14	13	12	7	5		15, 18	B(22)
	Tricaprylin	1965		2/104	0/10.4	C	10	7	7	6	5	5	13	25	
	Tricaprylin	1965		3/10	0/1.0	S	29	29	25	20	6	4		24	
	Tricaprylin	1968		1/26	0/2.6	S	16	16	14	14					
	None	1965				S	24	23	23	22	17	13		24	B(12)
	None	1965				S	100	80	66	56	51	21	14	14, 17, 17, 17, 17	B(15, 14, 15, 17, 17, 17, 17, 17, 15, 18), C(14, 15);
	None	1965				S	47	45	43	13	8	3		18	B(11, 12, 16, 16);
	None	1965				C	26	31	29	27	23	12		23	L(6, 23, 23, 27);
	None	1967				S	32	23	23	14	13			12, 12, 12	
	Fatty acids and esters														
IV	Stearic acid	1964	1.0	2/82	82/8.2	C	10	8	8	8	8	7			
	Stearic acid	1964	0.5	2/114	57.0/10.4	C	10	10	10	10	10	9		21	Ad (21)
	Stearic acid	1964	0.05	2/114	5.7/10.4	C	10	9	8	6	5	4	6, 10, 10, 12	19	L(19)
	Stearic acid	1965	0.05	2/104	5.2/10.4	C	15	14	14	14	14	13		19	
	Stearic acid	1966	1.0	2/20	10/1	S	15	15	14	8	4	1			
	Stearic acid	1967	0.5	1/26	13/2.6	S	16	14	7	7	7	6			
	Stearic acid	1967	0.05	1/26	1.3/2.6	S	16	13	12	11	10	10			

[a] S, Swiss/Webster; C, BALB/c; B, breast carcinoma; U, uterine carcinoma or sarcoma; C, cutaneous carcinoma; L, leukemia lymphoma; Ad, adenocarcinoma; K, kidney carcinoma; G, gastric carcinoma; Sa, salivary gland carcinoma.

As a control test, Hieger injected s.c. an unspecified amount of stearic acid dissolved in olive oil into 51 mice. No sarcomas resulted from this treatment (288).

Foreign-body Reaction

In a study by Herting and Crain, an unspecified number of weanling male and female Holtzman rats were maintained on a diet containing 50% stearic acid while a control group was given 50% lard. Rats were sacrificed at 8-week intervals and subjected to gross examination. Portions of perigastric, perirenal, periadrenal, perigonadal, and subcutaneous fat were removed from each rat and examined microscopically. Gross examination of the viscera and body cavities revealed only a general fullness of the gastrointestinal tract with creamy colored food and an occasional mottled appearance of fat depots in the animals fed the stearic acid. Microscopic examination of the excised fat showed an unusual foreign-body type reaction in fat from the rats fed stearic acid. The lesion consisted of a multinucleated cellular reaction usually located within the fat cell membrane with nuclei arranged evenly

about the periphery of the fat cell or in clumps. In many instances, the material which occupied the center of the fat cell, appeared to have been partially dissolved, leaving delicate slit-like spaces which were radially oriented and suggestive of fatty acid crystals. The reactive process usually involved 2 or more adjacent fat cells and was separated from similar areas by uninvolved fat cells. No acute inflammatory reaction, evidence of recent hemorrhage, or birefringent material were associated with the fat reaction. The foreign-body reaction occurred within 8 weeks in rats fed stearic acid, but was not observed in the control rats (286).

Biochemical Aspects

I. Breakdown

The heating of fats can lead to oxidation, polymerization and decomposition which may result in a vast mixture of reaction products, including polymers, peroxides, aldehydes, ketones, epoxides, lactones and free fatty acids. The possibility of heated fats having carcinogenic properties has been extensively researched without producing a definite conclusion (23).

Kieckebusch et al. reported that the blowing of oils at high temperatures (180 degrees C) greatly increases their toxicity, probably due to the formation of oxypolymers. The preparation of food products containing tallow could possibly result in formation of similar compounds (408).

II. Absorption - Distribution

Feddie et al. reported that the apparent absorbability, by chicks, of beef tallow fed as 10-20% of the diet: Increased from 53% at one week of age to 80% at 12 weeks of age; was increased when 0.5% or more of dietary ox bile was added; was not dependent upon growth rate or feed intake; and was decreased by high dietary calcium and increased by reducing the calcium intake (203).

To determine the degree of utilization of tallow by chickens of various ages, Renner fed tallow at a level of 17.5% in a basal diet. The results of the test (see Table 1) indicate that the utilization of tallow by chicks improves with age up to 8 weeks and is steady thereafter (635).

TABLE 1.—*Utilization of tallow by chickens of various ages*

Age in weeks	Energy utilization: Metabolizable energy		Energy utilization: Percent of gross energy[1]		Absorbability[2]	
	Cal./gm.				%	
2 weeks	6.18		66		65	
	6.97	6.58[3]	75	70	74	70
4 weeks	6.45		69		74	
	6.69	6.57	72	70	74	74
6 weeks	7.15		77		80	
	6.69	6.92	72	74	70	75
8 weeks	7.46		80		83	
	7.18	7.32	77	78	80	82
38 weeks (adult hens)	7.15		77		80	
	7.23	7.19	77	77	82	81

[1] Metabolizable energy as a percentage of gross energy of tallow (9.34 Cal./gm.).
[2] Computed from dietary fat and total fecal fat, correcting for fecal fat from low-fat reference diet.
[3] Underlined values are averages of duplicate lots; individual replicate values are in the respective left columns.

Using an adiabatic calorimeter, Petersen and Vik-Mo determined the energy content of tallow to be 9.484 kcal/g. In a study with young, growing chickens they found the metabolizable energy of tallow to be 7.05 kcal/g. The digestibility coefficients determined directly and calculated from the metabolizable energy are 91.3 and 74.3%, respectively (601).

On the basis of studies using day-old chicks, Sibbald et al. calculated the metabolizable energy (ME) value of tallow when fed as 10 and 20% of the diet in diets containing 24.4 and 34.0% protein. In the low-protein diet, ME values of 6.02 and 7.24 cal/g were calculated for tallow at 10 and 20%, respectively. ME values of 6.79 and 7.69 cal/g were calculated for the tallow at 10 and 20%, respectively, in the high-protein diet (697).

The absorbability of beef tallow by chicks was found by Young to increase from 71.7% at 3-4 weeks of age to 73.8% at 7-8 weeks of age. The utilization (metabolizable energy as percent of gross energy) of tallow increased from 71.5% to 73.2% during this period. Hydrolysis of the tallow to yield free fatty acids caused reductions in absorbability and utilization to 68 and 61%, respectively (838).

On the basis of a study in which New Hampshire male chicks were fed tallow in a basal diet at a level of 12% for 6 weeks, March and Biely calculated the digestibility coefficient of tallow to be 73% (492).

The metabolizable energy values and utilization of various fats and fatty acids for the chicken have been determined by Renner and Hill (see Table 1 on following page). Hydrolysis of tallow to the form of free fatty acids reduced the utilization value from 67-76.2% to 47.7%. When stearic acid was fed singly to chicks at 4 weeks of age a negative utilization value was obtained, indicating that stearic acid was not only not utilized but it also inhibited the utilization of other dietary components (634).

In digestibility studies using mature male rats, Hoagland and Snider fed stearic acid mixed in the proportions of 5, 10, 15 and 25% with pure olive oil. The fat mixture constituted 5% of the diet. Stearic acid was very poorly absorbed at each level of intake, with calculated digestibility coefficients of 9.4, 13.3, 21.0, and 19.6% at the 5, 10, 15 and 25% levels, respectively (305).

The apparent and corrected digestibilities of beef tallow, when fed to newly weaned pigs at 5% of the diet, were calculated by Bayley and Lewis to be 65 and 85%, respecitvely. The digestible energy value of tallow was calculated at 7.940 kcal/g. In further tests in which stearic acid was fed at 5% of the diet, the apparent and corrected digestibility coefficients for stearic acid were found to be 40.7 and 60%, respectively (58).

Carlson and Bayley, after feeding young pigs a diet containing 10% tallow for a period of nine days, determined the digestibility coefficient of tallow to be 56% (107).

TABLE 2. Metabolizable energy values and utilization of fats and fatty acids

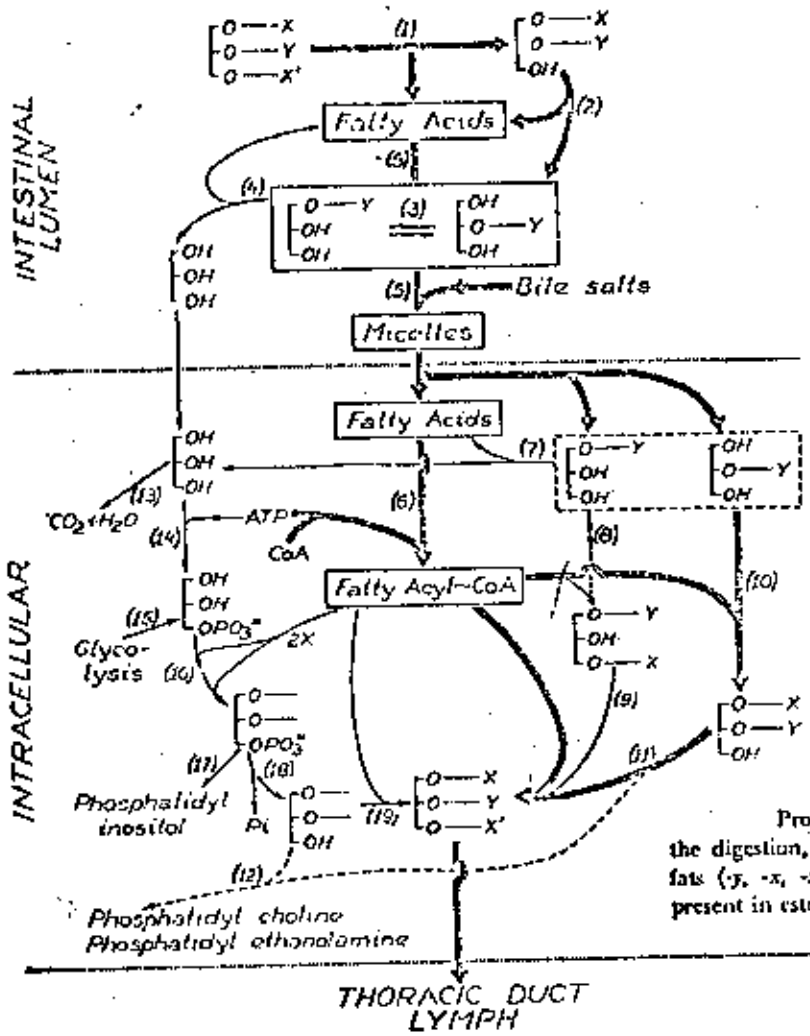
	Iodine number	M.E. Cal./lb.	Utilization %
Edible fats			
Corn oil	122	3950	92.9
Degummed soybean oil	133	4210	98.8
Lard	65	3980	93.4
Hydrogenated fat	69	3250	76.2
Inedible fats			
Feed grade tallow	52	2990**	70.8
		3230***	76.2
Beef tallow	44	2860	67.0
Hydrolyzed fat	61	3230	77.8
Menhaden oil	160	3700	87.8
Red fish oil	120	3580	83.4
Cod liver oil	154	3690	87.0
Fatty acids			
Palmitic		-9	-0.2
Stearic		-17	-3.9
Oleic		3770	88.4
Soybean F.A.	131	3750	89.3
Lard F.A.*	67	2730	64.8
Tallow F.A.*	44	2010	47.7
Miscellaneous			
Acidulated soybean soapstock	124	3150	76.3
Soybean lecithin		3060	86.5

* Derived by hydrolysis from the samples of beef tallow and lard studied.
** Young chicks, 2-4 weeks old.
*** Older chicks, 6-8 weeks old, and hens.

The digestibility of tallow when fed to calves at a level of 5% in an all-concentrate diet was found by Raven to be 87.6%. When hay was added to the diet so that the proportion of concentrates to hay was 2:1, the digestibility of the tallow increased to 90.5% (624).

According to Kayden et al. analysis of intestinal lumen contents of man after fat feeding has shown a separation of glycerides into two phases: an oil phase which contains mainly diglycerides and triglycerides and some fatty acid, and a micellar phase which is a combination of bile salts, free fatty acids, and monoglycerides. From the micellar solutions monoglycerides and free fatty acids are absorbed through the intestinal wall, leaving the bile salts within the lumen to form additional micelles. The fate of the free fatty acids within the mucosal cell is determined by their chain length and saturation; those of less than 12 carbons are preferentially absorbed by rats directly into the portal blood, whereas longer chain fatty acids are incorporated mainly into triglycerides, and appear in lymph chylomicrons. Monoglycerides within epithelial cells may be esterified to di- and triglycerides or cleaved to glycerol and free fatty acids. The 2-monoglycerides are esterified especially rapidly to triglycerides, but esterification of 1-monoglycerides lags and these are more likely to undergo hydrolysis particulary if the fatty acids are of short chain length (399).

In a review of the literature of fat absorption, Johnston stated that the mechanism of fat absorption can be summarized as shown in the following figure.



Proposed mechanism involved in the digestion, absorption, and biosynthesis of fats (-y, -x, -x', and —— denote fatty acids present in ester linkages).

The heavy arrows denote the major pathway. The emulsified triglycerides are hydrolyzed by pancreatic lipase, at the 1 and 3 positions, forming monoglycerides and fatty acids which in combination with bile salts form a micellar dispersion (reaction 5). The monoglycerides and fatty acids form micelles which penetrate the lumen membrane by an energy-independent mechanism, presumably by diffusion (reaction 5). The monoglycerides and fatty acids migrate to the endoplasmic reticulum, where the fatty acids are activated to their CoA derivatives (reaction 6) and are resynthesized into triglycerides by the acylation of monoglycerides in a multienzyme complex (reactions 10 and 11) (376).

III. Metabolism and Excretion

Fatty acids play an important role as an energy source. Their

oxidation is prominent in higher animals which can store large amounts of neutral fat as a fuel reserve. It is estimated that fatty acid oxidation provides at least half of the oxidative energy in the liver, kidneys, heart muscle and resting skeletal muscle. 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 triacylglycols but very small amounts of free fatty acids. The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consists largely of triacylglycerol. Before the fatty acids can undergo activation and oxidation, the triacylglycerols first must undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerols. Free fatty acids are therefore derived either from the blood stream, in which they are transported bound to serum albumin, or prior hydrolyses of intracellular lipids. They are first activated by esterification with CoA to form acyl CoA esters of the outer mitochondrial membrane. Then the fatty acyl group from CoA is carried across the inner membrane by the carrier molecule carnitine. Finally the fatty acyl group is transferred from carnitine to intramitochondrial CoA: through several enzymatic reactions. A two-carbon fragment of the fatty acid is removed as acetyl CoA and two pairs of hydrogen atoms are given to specific acceptors. A long chain fatty acid will go through several spirals. Thus the 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules of acetyl CoA and 14 pairs of hydrogen atoms. The acetyl CoA formed as a product of the fatty acid oxidation will then enter the tricarboxylic acid cycle, which is the final common pathway of oxidation catabolism of all fuel molecules in aerobic cells (452).

IV. Effects on Enzymes and Other Biochemical Parameters

Lin et al. observed that stearic acid fed to chickens at a 10% level by weight in the diet resulted in a significant increase in the rate of hepatic cholesterol synthesis above that of the control group (465).

Male albino rats of the Holtzman strain were observed by Janssen to exhibit a rise in plasma cholesterol when fed a diet containing 15% free stearic acid (365).

Dietary studies by Hegsted et al. using 21 middle-aged male mental patients indicate that stearic acid plays a significant role in elevating serum cholesterol (280). However, Karvinen et al. (387) reported that oral administration of 30 g of stearic acid per day to 6 young healthy adult humans did not significantly influence the absorption of dietary cholesterol and Grande et al. (241) concluded on the basis of their studies that stearic acid has no effect on the serum cholesterol concentration in man.

Renaud and his co-workers reported that stearic acid and tallow appear to be thrombogenic, stearic acid much more so than tallow, when included in the hyperlipemic diet of rats, since they markedly predispose the animals to the production of large, occlusive, endotoxin-

initiated thromboses. Stearic acid was also found to exhibit a strong atherogenic effect (632, 629, 630).

In vitro studies have shown that stearic acid enhances mitochondrial ATPase and swelling (487, 791, 844).

The feeding of a diet containing 15% tallow to miniature pigs for 1 year was found by Mahadeven et al. to significantly decrease the blood clotting time 3 hours after ingestion of the fat (484).

Stearic acid produced constipation in dogs when mixed with a standard dog food at a level of 5% (823).

V. Drug Interaction

Ehrenstein and Corey reported that stearic acid increased the effect of testosterone upon the accessory sex organs of the castrated male rat (186).

VI. Consumer Exposure Information

Tallow is used in the manufacture of margarine, and as a defoamer component. Potassium stearate is also used as a defoamer component while sodium stearate is used as an emulsifier, binder and chewing-gum base component. Magnesium, calcium and sodium stearates are used as anti-caking agents in onion and garlic salts, and meat dry-curing mixtures. There is no information available from the NAS/NRC Questionnaire.

TALLOW

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HEATED FATS AND ALLIED COMPOUNDS AS CARCINOGENS

Studied by the Newt Test

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Received 4.x.63

Extensive studies through decades have failed to clarify whether heated and oxidated fats have carcinogenic properties. This appeared from a review of accumulated experimental results (*Arffmann* 1960) pointing to the incapability of the conventional methods of bioassay to yield conclusive evidence. It seems therefore highly desirable to develop new methods for the demonstration of carcinogenicity which at the same time may be expected to reveal unknown effects of carcinogens. A series of experiments on the newt test (*Arffmann & Christensen* 1961, *Arffmann* 1962, 1963 and 1964) has shown a specificity sufficient for a screening method, if the chemical compounds are given in oil, which makes the method convenient for the study of heated fats and allied compounds.

Heating and Oxidation of Fats.

The chemical changes induced by heating of lipids depend on the presence or absence of oxygen, the active components being primarily the unsaturated fatty acids (*Axt* 1959). Most important is the oxidative transformation, corresponding to autoxidation which has found excellent description in works by *Paquot* (1958), *Axt* (1959) and *Glavind* (1963). The first step is the formation of hydroperoxides (lipoperoxides) formed by a free-radical mechanism and proceeding as a chain reaction. This is terminated by polymerization. Decomposition of the peroxides leads amongst others to a formation of hydroxyl-, aldehyde- and ketone groups as well as acids, and the products include a series of low-mole-

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cular-weight unsaturated carbonyl compounds. Also epoxides are formed late in the process.

If oxygen is absent during heating oxygen-free polymers, often cyclic structure, are formed. Decomposition products are paraffins, aldehydes, acids and ketones.

The most simple analytical methods to follow the oxidation are determinations of the peroxide value and of the carbonyl (aldehyde) value. The peroxide value expresses the actual quantity of hydroperoxide groups and is therefore not a measure of the total oxidation (*Deutsch et al.* 1941). The carbonyl value measures the secondary oxidation products, which are determined as aldehydes (*Holm et al.* 1957).

The present knowledge of the chemical changes in heating and oxidation of fats is, however, only elementary. In summary, complicated processes of oxidation, polymerization and decomposition lead to a vast mixture of reaction products, including polymers, peroxides, aldehydes, ketones, epoxides, lactones and free fatty acids (*Wurziger & Osterberg* 1960).

Heating of cholesterol probably leads to oxidation, and the formation of ketonic steroids is suggested by *Haven & Bloor* (1956). The products may possess hydroperoxide- or epoxide groups (*Fieser* 1954).

MATERIAL AND METHODS

Substances. Soyabean oil was chosen as test object for the study of heated fats. This oil is used for the preparation of edible emulsifiers, especially for the manufacture of margarine and baking compounds, and besides its content of the more unsaturated fatty acids is rather high.

Simple heating was performed on an infrared radiation bath (BB Infrabath, Bie & Berntsen, Copenhagen) furnished with a relay and a contact thermometer. The fresh soyabean oil (supplied by Dansk Sojakagefabrik) was contained in a Pyrex beaker or retort and the contact thermometer put into the oil. A temperature of 350° C was reached after 45 to 60 minutes and maintained for exactly ½ or 1 hour. During this time the temperature was not below 350° C and not above 355° C. After the heating period the oil was immediately removed from the warm plate. Air had free admittance, but was not bubbled through. The end-product was a dark, brownish and distinctly viscous material.

Some of the soyabean oil was heated in nitrogen atmosphere. The fresh oil was bubbled through with nitrogen for 6 hrs. 20 min. before being poured into a retort, which was sealed from the surrounding air. The warm-up time was 105 minutes and a strong current of nitrogen through the retort was maintained until 300°–320° C was reached after about 70 minutes. The sealed system was heated at 350°–355° C for exactly 1 hour. The nitrogen current was reestablished during the first hour of cooling, and then the retort was closed. The end-product was of a lighter colour and less viscous than that after heating in the presence of air.

Chemical data of the polymerized soyabean oils are given in Table 1.

The viscosity of the heated oils made it necessary to dilute the products with 3 parts of fresh soyabean oil (v/v) before injection.

Commercially polymerized soyabean oil was kindly supplied by Grindstedvaerket, Viby J., Denmark. It is used in an edible emulsifier and produced by heating at 300° C in vacuum (about 20 mm Hg) for about 48 hours until the desired viscosity has been reached. Chromatographic analysis (performed by Professor Jacini, Milan) after saponification of the oil showed the following approximate percentages: monomeric acids: 43–50, dimeric acids: 32–38, polymeric acids: 15–16.5. Molecular weight

of the monomeric glyceryl fraction was about 920, while total oil molecular weight was 2470–3120. Because of the viscosity the product was administered as a 25 per cent in one experiment 50 per cent solution in fresh soyabean oil (w/w).

TABLE 1

Chemical Analyses of Heat-Polymerized Soyabean Oil.

Substance	Peroxide value m.e./kg	Carbonyl value mg%	Iodine value	% Urea filtrate Acids	% Non-distillable residual waters
Soyabean oil	1.4	6.2	131	6	5
Soyabean oil heated in N-atm. at 350° C for 1 hr.	4.7	217	75	58	53
Soyabean oil heated in air at 350° C for 1 hr.	5.1	206	73	66	56
Soyabean oil heated in air at 350° C for ½ hr.	...	...	...	...	51

The peroxidized soyabean oils were prepared by the Laboratory at Grindstedvaerket. Moderate heating, vigorous stirring of the oil and a constant current of air through the retort were maintained until the peroxide content had risen to the highest obtainable values. Before that, specimens had been taken out at lower levels with proper intervals. In experiments Nos. 11 and 12 (Table 3) the P.V.[1] 4.5 oil was oxidated at 37° C for 131 hours, while the P.V. 858 specimen was made at 100° C for 21 hours. The same temperature was applied to the P.V. 293 oil through 28 hours, but after the oxidation this specimen was mixed with equal part of fresh soyabean oil (the peroxide value is determined on the mixture). In spite of several trials it proved impossible once more to reach the high peroxide value of 8–900 m.e./kg. The applied temperatures were 70° C and down to 40° C in order to avoid heat-induced reversion of the process which ran for up to 95 days. The highest peroxide value obtained in these latter experiments was 409 m.e./kg.

For parallel control, specimens of the peroxidized oils were quickly heated at 200° C for 10 minutes at 10 mm Hg in order to destroy the peroxides. These samples are designated B in the tables.

The peroxidized soyabean oils were—with a few exceptions—injected without any dilution.

Ethyl linoleate hydroperoxides were kindly prepared by Johs. Glavind, D.Sc., Polytechnic Institute, Copenhagen, who also supplied the ethyl linoleate. The technique of preparation is described by *Glavind, Sandegaard & Dam* (1961). The peroxide values (*Glavind & Hartmann* 1955) of the three specimens were 2000, 1500 and 750 m.e./kg. Part of the P.V. 1500 sample was reduced following the method of *Privett & Nickell* (1956), and the peroxide value thereby lowered to 70 m.e./kg. The reduction lasted 1 hour. In most experiments the ethyl linoleate preparations were solved in peanut oil to reduce toxicity, concentrations being v/v.

Epoxidized soyabean oil was obtained from General Mills, U.S.A., and is designated Epoxygen (type 90 premium grade). The following analytical values are specified: Epoxy content, per cent of theoretical: 90 per cent min. Oxirane content: 6.8 per cent min. Iodine value (Wijs): 2.0 max. Acid value: 0.2 max. Saponification value: 180–184. Molecular weight: 1000 appr. Because of viscosity the epoxygen had to be diluted with fresh soyabean oil before injection to make a 25 per cent solution (v/v).

The various isomers of diepoxybutane (*Feit* 1966) were kindly supplied by Dr. P. W. Feit, the Leo Pharmaceutical Products, Copenhagen. The pure d-, l-, meso- and dl- compounds were all easily solved in peanut oil without the use of heat. The concentrations are weight/volume except in experiment No. 65 (v/v).

16 α, 17 α-epoxyoestratrien-3-ol, synthetisized and kindly provided by Professor H. Breuer, Bonn, was tested in the newts. This monoepoxide, which is a naturally

[1] P.V. — peroxide value, determined a.m. Wheeler (DGF Einheitsmethoden C-VI 6a (67)).

occurring product in estrogen metabolism, is closely related to oestradiol, which was chosen for parallel control and kindly supplied by the Leo Pharmaceutical Products. Both substances were found difficult to solve in peanut oil requiring vigorous stirring and heating on waterbath (<100° C) for 5 to 11 minutes to obtain 0.5 per cent solutions. In most instances slight secondary precipitation after cooling was eliminated before injection by heating over open fire for a few seconds.

Cholesterol was obtained from The British Drug Houses Ltd. (Laboratory Reagent. Melting point: 145° to 149° C. Specific rotation (α) $\frac{20}{D}$ (in chloroform): −38°.5 to −40°.5. Moisture: Not more than 0.5 per cent. Sulphated ash: Not more than 0.1 per cent). Cholesterol was heated on the BB Infrabath the compound being placed in a Pyrex glass, which together with the contact thermometer was put into a beaker with silicon oil. One sample was heated at 350° C, another at 200° C, both for exactly 1 hour. The warm-up time was 30 and 14 minutes, respectively. After the 350° C heating cholesterol appeared darkbrown to black and after cooling was paste-like. The lower temperature changed the white colour to light yellow-brown. Both specimens were, like untreated cholesterol, easily solved in oils by the use of stirring and heating on waterbath (<100° C) for 1–1½ minutes.

Technical procedures have been described before (*Arffmann & Christensen* 1961, *Arffmann* 1962, 1963). In a few experiments Triton marinoratus was used instead of Triton cristatus, the latter being at that time not available.

RESULTS

The results are shown in Tables 2-10. Negative and positive controls are included. The various heat-polymerized soyabean oils gave a negative outcome in all experiments (Table 2).

Strongly peroxidized soyabean oil was submitted to two tests (Table 3). The results are slightly varying, probably because of a less reliable technique in these early experiments, but indicate clearly a weak or moderate, positive reaction to the two oils with a peroxide value of 858 and 293 m.e./kg respectively. In contrast, the P.V. 4.5 oil gave a result not different from that seen after fresh oil, while heavy dilution (2 per

TABLE [illegible]

Experiments with Heat-polymerized Soybean Oil.

Experiment No.	26-44					8						16	
Substance	Soybean oil	Soybean oil heated in air at [illegible] for [illegible] hr., 50% in soybean oil	Ethylene [illegible] 0.1% in soybean oil	Soybean oil		Soybean oil heated in [illegible] at [illegible] for 1 hr. 25% in soybean oil		Soybean oil heated in air at 250° for 1 hr. 25% in soybean oil		Soybean oil		Soybean oil heated in [illegible] at [illegible] for 1 hr. 25% in soybean oil	
Site of application	Tail		Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back
Number of animals (Yellow-mice)	4	8	8	8	4	4	4	4	8	4	4	8	1
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal tumor first seen													
3–4 days													
5–7													
8–10													
11–12													
15													
16													
Animals died during experiment	0	1 (un-charted)	3 (2 [illegible] charted)	1	4	1 ([illegible] charted)	1	2	0	3	4	3 (charted)	4

Experiment No.	16		17					30			31			32	
Substance	Soybean oil heated in air at 250° for 1 hr. 25% in soybean oil		Soybean oil	Soybean oil heated in [illegible] at 250° for 1 hr. 25% in soybean oil	Soybean oil heated in [illegible] at 250° for 1 hr. 25% in soybean oil	Benzoyl peroxide 0.5% in soybean oil	Soybean oil	Commercially polymerized soybean oil 25% in soybean oil	Benzoyl peroxide 0.1% in soybean oil	Soybean oil	Commercially polymerized soybean oil 25% in soybean oil	Benzoyl peroxide 0.1% in soybean oil	Soybean oil	Commercially polymerized soybean oil 25% in soybean oil	Charcoal 0.5% in soybean oil
Site of application	Tail	Back	Tail					Tail			Tail			Tail	
Number of animals (Yellow-mice)	1	4	8	8	8	8	4	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal tumor first seen															
3–4 days															
5–7															
8–10															
11–12															
15															
16															
Animals died during experiment	1	1	1	2 (un-charted)	2 ([illegible] charted)	2 (un-charted)	4	2	1 (un-charted)	1	1	2 (un-charted)	0	4	0

* Omitted because of epidermolysis.

TABLE 2

Experiments with strongly peroxidized Soyabean Oil.

TABLE 2

Experiment No.	11								12							
Substance	Peanut oil	Perox. soya-bean oil (P.V.* 4.5)	Perox. soya-bean oil (P.V. 290)	Perox. soya-bean oil (P.V. 858)	Perox. soya-bean oil (P.V. 4.5) 2% in peanut oil	Perox. soya-bean oil (P.V. 290) 2% in peanut oil	Perox. soya-bean oil (P.V. 858) 2% in peanut oil	3-Methyl-cholanthrene 0.1% in peanut oil	Soya-bean oil	Perox. soya-bean oil (P.V. 4.5)	Perox. soya-bean oil (P.V. 290)	Perox. soya-bean oil (P.V. 858)	Perox. soya-bean oil (P.V. 4.5) 2% in soya-bean oil	Perox. soya-bean oil (P.V. 290) 2% in soya-bean oil	Perox. soya-bean oil (P.V. 858) 2% in soya-bean oil	3-Methyl-cholanthrene 0.1% in soya-bean oil
Site of application	Tail								Tail							
Number of animals (Triton cristatus)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																
3. day	–									(+)	–	+	–		+	
6. "			(+)	–				(+)			–	+	–	–	+	–
9. "	–		+		–		(+)			+	–	+	–	–	–	+
12. "	–				+				–	–		+	–	–	–	(+)
15. "	–	–	+		–				–			–	+	–	(+)	+
18. "		–	–	+	–			+		+	+	–		–	+	–
21. "	–	–			–	–	–		–	–	–	+	–		–	
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* P.V. = peroxide value (m.e./kg).
† No signs of the injected oil.
‡ Lesions technically bad.

TABLE 4

Experiments with Peroxidized Soyabean Oil before (A) and after (B) Heat-Reduction.

Experiment No.			45	46	47	49	50	51	52	54	69	70
Substance	P.V.*	C.V.§	Positive reactions/Total number of animals									
Oil 0	1.9	12.5	1/5	0/5	0/6	...	...	...	...	...	...	...
Oil 0	2.2	3.7	...	...	...	0/8	0/8	0/7	0/8	...	...	...
Oil 0	1.4	10.2	...	...	...	...	...	...	...	1/6	...	...
Oil 0	3.0	2.5	...	...	...	...	...	...	...	...	1/8	0/8
Oil 1A	33.0	9.5	0/7	0/6	...	...	...	...	...	...	...	...
Oil 2A	55.0	...	...	...	...	0/8	...	...	...	0/6	...	...
Oil 3A	70.0	9.1	0/6	0/6	...	...	...	...	...	...	...	...
Oil 4A	104.0	...	...	...	...	0/8	...	...	...	0/6	...	...
Oil 5A	161.0	13.1	2/7	1/6	...	...	...	...	...	...	...	...
Oil 6A	191.0	...	...	...	...	0/8	...	1/8	...	0/6	...	...
Oil 7A	296.0	22.0	0/2	0/5	2/4	...	...	...	...	...	...	...
Oil 8A	362.0	...	...	...	...	0/8	0/8	0/8	0/8	0/6	...	...
Oil 9A	409.0	57.2	...	...	...	...	...	...	...	...	2/8	3/16
Oil 1B	1.5	47.0	0/3	0/5	...	...	...	...	...	...	...	...
Oil 2B	0.6	67.0	...	...	...	0/8	...	...	...	0/5	...	...
Oil 3B	1.8	90.0	0/6	2/5	...	...	...	...	...	...	...	...
Oil 4B	0.7	137.0	...	...	...	0/8	...	...	...	0/5	...	...
Oil 5B	2.6	162.0	0/6	0/6	...	...	...	...	...	...	...	...
Oil 6B	1.3	310.0	...	...	...	0/8	...	0/7	...	0/6	...	...
Oil 7B	4.1	165.0	0/3	0/6	0/4	...	...	...	...	...	...	...
Oil 8B	3.8	640.0	...	...	...	1/8	0/7	0/8	0/8	0/6	...	...
Oil 9B	3.1	336.0	...	...	...	...	...	...	...	...	2/7	3/18
Dibenz(a,h)-anthracene 0.1%			2/2	...	...	...	...	...	...	...	...	...
Chrysene 0.5%			...	2/5	...	...	...	...	3/8	...	...	...
Benzo(a)-pyrene 0.1%			...	...	3/6	...	1/8	4/8	...	...	5/7	...
0.5%			...	...	...	...	...	...	...	...	...	8/8
Benz(a)-anthracene 0.1%			...	...	...	0/6	...	...	...	...	...	...
0.5%			...	...	...	...	...	...	...	2/5	...	...

* P.V. = peroxide value (m.e./kg).
§ C.V. = carbonyl value (mg%).

cent) of the P.V. 858 and 293 oils weakened the positive response distinctly.

The following extensive experiments on the reactivity of the newt epidermis to peroxidized soyabean oil are summarized in Table 4. The oil numbering is arbitrary and in series A refers to the increasing peroxide value of the samples, while series B represents the same specimens after heat destruction of the peroxides. O oils are untreated, fresh soyabean oils. Positive reactions cover + and (+) responses, while doubtful reactions (±) are not included. Total number of animals represents effective total. Eleven positive reactions to soyabean oils with a peroxide value of 161 or more were seen among 108 newts. With one exception all of these responses fall in experiments 45–47, being made in the autumn, and in experiments 69–70, using hibernating animals from the preceding year's batch, which in both cases means that

Table 5.

Experiments on a Possible Cocarcinogenic Effect of Oxidized Soybean Oil.

Experiment No.	[illegible]
Substance	[illegible]
Site of application	Tail / Tail, Pr. T. [illegible]
Number of animals (Triton cristatus)	[illegible]
Sex	[illegible]
Epithelial reaction on	
3.–4. day	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
Animals died during experiment	[illegible]

* Numbering of the oils is the same as in Table 4.
P.V. = peroxide value (m.e./kg). C.V. = Carbonyl value (mat%).
§ Triton marmoratus in exper. no. 47.
** Omitted because of heavy skin-infection.
Part of tail produced by Secondary tail.

the animals have been in a rather sensitive state (cf. *Arffmann* 1964). Experiments 49–54, holding only one positive response, were made in the spring on newly received and therefore more resistant animals. This is reflected by the positive controls in experiments 49–50. In the B series the result of experiments 69–70 equals that in the A series. Besides, the positive reactions are few, but it is difficult to explain why 2 out of 5 animals in experiment No. 46 react to an oil with carbonyl value 60 considering the many negative responses at higher carbonyl values.

A possible cocarcinogenic effect of oxidized soybean oil was examined in three experiments shown in Table 5. The first experiment (No. 47) was indicative of such an effect, but could not be confirmed. Unfortunately an unexpected high reactivity to benzpyrene was seen at a concentration of 0.025 per cent, and signs of promotion could not be demonstrated, when oxidized soybean oil was used as vehicle instead of the fresh oil.

The first experiment with ethyl linoleate hydroperoxide is not tabulated. Since the preparation of the compound several months earlier the peroxide value had fallen from 3000 to 2000 m.e./kg. The oil was injected undiluted into 11 Triton marmoratus and 1 Triton cristatus. From the 4th day severe toxic lesions with extensive necrosis and deep ulceration were seen in the tails, so that the experiment had to be terminated within 9 days. The lesions were grossly like those seen after tributyrin (*Arffmann* 1962), but microscopically the epithelial reaction was of quite different degree with extensive and strong hyperplasia. Less striking was the slight or moderate infiltrative downgrowth at the edges or in the bottom of the ulceration. The severe inflammatory lesions made it impossible to estimate the epithelial reaction exactly in terms of degree, but the changes strongly indicated further experimentation.

Tables 6 and 7 show the results with ethyl linoleate hydroperoxide of peroxide value 750 and 1500 m.e./kg and in various concentrations, compared to the effect of reduced ethyl linoleate hydroperoxide and pure ethyl linoleate. The degree of toxic symptoms was practically equal

TABLE

Experiments with Ethyl Linoleate Hydroperoxide (High Concentrations).

Experiment No.	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Substance	Sesame oil	Ethyl linoleate hydroperoxide [illegible]	Ethyl linoleate hydroperoxide [illegible]	Reduced [illegible] in sesame oil	Sesame oil	Ethyl linoleate	[illegible]	Peanut oil	Ethyl linoleate hydroperoxide [illegible] in peanut oil	Ethyl linoleate hydroperoxide [illegible] in peanut oil	[illegible]
Site of application	Tail				Tail					Tail	
Number of animals (Triton criterion)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on											
4–7 day	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
8–11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible] during experiment	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Substance	[illegible] in peanut oil	Peanut oil	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Peanut oil	[illegible]	[illegible]	[illegible]	[illegible] in peanut oil
Site of application				Tail						Tail			Tail
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀

* Severe necrosis. † Sections technically bad.

For the two peroxides (Fig. 1) and the reduced form, but decreased with concentration, being absent at 10 per cent. Undiluted ethyl linoleate had negligible toxicity. A few cases (in experiments 54 and 55) could not be classified because of severe necrosis. Ethyl linoleate hydroperoxide P.V. 1500 induced a strong, positive reaction in the newt when undiluted and as a 50 per cent solution in peanut oil (Fig. 2). The response decreased with the concentration and was totally negative at 1 per cent. The reaction to the reduced compound was a little less, but also positive. Ethyl linoleate hydroperoxide P.V. 750 induced a clearly weaker positive response than the P.V. 1500 compound, and pure ethyl linoleate can be estimated as negative.

The toxic lesions being similar to those observed after the injection of tributyrin made it interesting to compare the latter compound with ethyl linoleate hydroperoxide in a few experiments. Toxicity showed to be of the same order. In one experiment [illegible] a 50 per cent solu-

[illegible] of tributyrin in peanut oil had the same carcinogen-like effect as ethyl linoleate hydroperoxide P.V. 750, and even at 10 and 1 per cent single positive reactions were seen. This result seems to indicate that tributyrin is not a totally indifferent compound (cfr. [illegible]). Peroxide value of the tributyrin was 0 and carbonyl value 1.4.

A completely negative result followed the injection of epoxidized [illegible] oil in three experiments (Table 6). The same applies to [illegible] which was submitted to three separate tests [illegible] animals with intact tails. Also untreated [illegible] negative, while [illegible] per cent benzo(a)pyrene in the positive control experiments [illegible] epithelial proliferation in [illegible] out of [illegible] animals. In contrast, the testing of a low-molecular [illegible] [illegible] function in all [illegible] (Table 6). A preliminary [illegible] with [illegible] revealed a [illegible] irritative

[illegible]

TABLE 2

Experiments with Ethyl Linoleate Hydroperoxide (Low Concentrations).

Experiment No.	30							30										
Substance	Present oil	Ethyl linoleate 10% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in present oil	Present oil	Triboxyrin 1% in present oil	Triboxyrin 10% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in present oil	Response to 0.5% in present oil	Present oil	Ethyl linoleate 1% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in present oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in present oil	Response to 0.5% in present oil
Site of application	Tail								Tail								
Number of animals (Triboxyrin crystals)	6	6	6	6	6	6 (Tr. crist. & vulg.)	6		6	6	6	6 (Tr. crist. & vulg.)	6	6	6	6	6
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
1.–3. day																	
8.–9.																	
13.–15.																	
14.–16.																	
18.–20.																	
Animals died during experiment	0	0	0	0	0	0	0		1	1 (Tr. crist.)	0	0	0	0	0	1 (Tr. crist.)	0

effect at a concentration of 10 per cent, but at 1 and 0.5 per cent such lesions were not seen. In two out of three experiments the positive reaction to the meso-compound was slight, but else there was no significant difference between the various isomers (Fig. 3).

In seven different experiments 0.1 or 0.5 per cent solutions of cholesterol induced no epithelial reaction in newts. After heating of the cholesterol at 200° C for 1 hour, one single positive response occurred among 16 animals, injected with 0.5 per cent solutions. Preheating at 350° C, however, formed a product which in the same concentration provoked epithelial reaction in about half the animals (Fig. 4). Experiment No. 06 confirmed this finding, being made 7 months later. A 0.1 per cent solution of the heated cholesterol had no effect. (Table 10).

DISCUSSION

The present results with peroxides suggest a weak carcinogenicity of such compounds. Most impressing was the reaction to ethyl linoleate hydroperoxide at high peroxide value and in high concentrations, while peroxidized soybean oil had a weak and inconstant effect. This difference parallels the variation in peroxide value, indicating a causality which is further supported by experiments Nos. 11 and 12, and by the quantitatively different response to the two ethyl linoleate hydroperoxides (P.V. 1500 and 750). The results may, however, have been influenced by the fact that the linoleate peroxide is a low-molecular, more diffusible and therefore immediately active compound, while the peroxy groups in the soybean oil are connected to larger and less mobile molecules. The severe toxic reaction to ethyl linoleate hydroperoxide is another expression of this circumstance and may have added to the

epithelial response. The carcinogenicity of peroxides is considered weak, because positive reaction demands a very high peroxide value of the oil or the use of sensitive animals.

The positive results obtained with reduced peroxides indicate that the activity is connected with other oxidation products as well, even if peroxy groups seem to be the most active. The carbonyl value is a summarized expression of the secondary oxidation products in the thermo-reduced soyabean oils (B series), while reduction of ethyl linoleate hydroperoxide probably leads to the formation of alcohol groups (*Glavind* 1963). The exact nature and quantities of these various reaction products is not known.

That the observed effect is connected with oxidation of the oils is supported by the other results of the present experiments. Heating of cholesterol is supposed to yield oxidative transformation products (*Fieser* 1954, *Haven & Bloor* 1956), and cholesterol heated at 350° C provoked a positive response in newts. Diepoxybutane likewise showed a distinct activity, while the negative result with epoxidized soyabean oil may be explained by the high molecular weight and low mobility of the active groups. The results with 16 α, 17 α-epoxyoestratrien-3-ol may express inactivity of monoepoxides.

Commercially polymerized soyabean oil is heated in vacuum, and the soyabean oil heated at 350° C in the presence of air showed only a mild degree of oxidation, the carbonyl value not exceeding that seen after heating in nitrogen atmosphere. Also *Wurziger & Osterlag* (1960) found a moderate rise in carbonyl value on heating of oils in a current of nitrogen. Table 4 shows that with a few exceptions oils in series B with a carbonyl value below 300 gave no positive reactions. Furthermore, the high-molecular-weight of polymerized oils will restrain the effectivity of any oxidized groups. So the negative effect of heat-polymerized soyabean oil is in accordance with the hypothesis that oxidation is the major factor responsible for the carcinogen-like activity of some heated lipids in newts.

Figs. 1-4.

Fig. 1. Large ulcerative defect with necrotic tissue remnants on 12th day after injection of ethyl linoleate hydroperoxide (P.V. 1500), 50 per cent in peanut oil. Strong epithelial hyperplasia is seen at the edge of the ulceration and on the other side of the fall. (10 ×).

Fig. 2. Epithelial hyperplasia and infiltrative downgrowth on 19th day after injection of ethyl linoleate hydroperoxide (P.V. 1500), 50 per cent in peanut oil (40 ×).

Fig. 3. Strong epithelial hyperplasia and infiltrative downgrowth on 15th day after injection of 1-diepoxybutane, 1 per cent in peanut oil. (40 ×).

Fig. 4. Epithelial hyperplasia and downgrowth on 18th day after injection of heat-cholesterol (350° C for 1 hour), 0.5 per cent in peanut oil. (40 ×).

Fig. 1–6.

TABLE 8

Experiments with Epoxidized Soyabean Oil.

Experiment No.	39						40				41						
Substance	Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil		Benzpyr-anthracene 0.1 % in soyabean oil		Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil		Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil				
Site of application	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back			
Number of animals (Triton cristatus)	4	4	4	4	4	4	4	4	4	4	4	4	4	4			
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
5. day		–				+											
8. -	–		–	–	–			–	–	–	–	–		–			
11.-12. -	+	–	–	–			–	–	–	– –	–	–		–			
15. -	–				–	–		–	–	–	–	–		–			
18. -	±		–	–	+	–		–	–		–	–		–			
Animals died during experiment	0	0	0	0	0	1 (included)	1	0	0	2 (included)	0	0	3	0			

TABLE 9

Experiments with Diepoxybutane.

Experiment No.	65		66					67					68					
Substance	dl-Diepoxybutane 10% in peanut oil	dl-Diepoxybutane 1% in peanut oil	Peanut oil	d-Diepoxybutane 0.5% in peanut oil	l-Diepoxybutane 0.5% in peanut oil	meso-Diepoxybutane 0.5% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil	Peanut oil	d-Diepoxybutane 1% in peanut oil	l-Diepoxybutane 1% in peanut oil	meso-Diepoxybutane 1% in peanut oil	Benzo-a-pyrene 1% in peanut oil	Peanut oil	d-Diepoxybutane 1% in peanut oil	l-Diepoxybutane 1% in peanut oil	dl-Diepoxybutane 1% in peanut oil	meso-Diepoxybutane 1% in peanut oil	Benzo-a-pyrene 1% in peanut oil
Site of application	Part of tail proximal to secondary tail		Part of tail prox. to secondary tail					Part of tail prox. to secondary tail					Part of tail proximal to secondary tail					
Number of animals (Triton cristatus)	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																		
6. day																		
9. "	(+)																	
12. "																		
15. "	(+)																	
18.-19. "																		
Animals died during experiment	0	0	0	0	0	0	0	0	8	0	8	0	8	8	0	0	3 (1 included)	1 (not included)

Experiments with Chol[illegible] and Heated Cholesterol.

Experiment No.	[illegible]		[illegible]							[illegible]			
Substance	Peanut oil	Cholesterol [illegible] in peanut oil	Soyabean oil	Cholesterol [illegible] in soyabean oil	[illegible] in soyabean oil	Soyabean oil	Cholesterol [illegible] in soyabean oil	Cholesterol heated at [illegible] in soyabean oil	[illegible] in soyabean oil	Peanut oil	Cholesterol [illegible] in peanut oil	Cholesterol heated at [illegible] in peanut oil	[illegible] in peanut oil
Site of application	Tail	Part of tail grown in secondary tail				Tail				Tail			
Number of animals (Triton cristatus)	6	6	6	6	8	8	8	8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Sex	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Epidermal reaction													
[illegible] day	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Animals died before expiration	[illegible]	[illegible]	[illegible]	0	[illegible]	[illegible]	0	0	0	1	[illegible]	[illegible]	0

Experiment No.	[illegible]				[illegible]			[illegible]				[illegible]			
Substance	Peanut oil	Cholesterol [illegible] in peanut oil	Cholesterol heated at [illegible] in peanut oil	[illegible] in peanut oil	Peanut oil	Cholesterol heated at [illegible] in peanut oil	[illegible] in peanut oil	Peanut oil	Cholesterol [illegible] in peanut oil	Cholesterol heated at [illegible] in peanut oil	[illegible] in peanut oil	Peanut oil	Cholesterol [illegible] in peanut oil	Cholesterol heated at [illegible] in peanut oil	[illegible] in peanut oil
Site of application	Tail				Part of tail grown in secondary tail			Tail				Tail			Back
Number of animals (Triton cristatus)	8	8	8	8	[illegible]	8	[illegible]	[illegible]	[illegible]	8	8	[illegible]	[illegible]	8	8
Sex	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Epidermal reaction															
[illegible] day	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Animals died before expiration	[illegible]	0	0	[illegible]	[illegible]	1	0	[illegible]	[illegible]	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

* Minimal signs of the injected oil.

SUMMARY

Some heated and oxidated fats and a few allied compounds were tested in newts. Positive results of varying degree were obtained with peroxidized soyabean oil and ethyl linoleate hydroperoxide, but the carcinogen-like activity of the peroxides is considered rather weak, when the response is compared with the magnitude of peroxide values and the sensitivity of the animals. Reduction of the peroxides through heating or by chemical means weakened the activity only slightly. Epoxidized soyabean oil and 16 α, 17 α-epoxyoestratrien-3-ol induced no epithelial reaction, while the testing of diepoxybutane proved positive for all isomers. Heating of cholesterol at 350° C for 1 hour gave a newt-positive product, while untreated cholesterol and moderately heated cholesterol (200° C for 1 hour) were negative. Commercially polymerized soyabean oil and soyabean oil heated at 350° C for ½ or 1 hour had no effect on the skin of newts. It is concluded that oxidation may be considered the most important process in the transformation of lipids leading to newt-positive and therefore carcinogen-suspected products.

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Identification of Oils and the Detection of Oil Adulteration by Differential Infrared Spectroscopy*

By J. G. BARTLET and J. H. MAHON (Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada)

The sophistication of a high-priced food with similar but less expensive products is a problem that has existed for many years. This type of adulteration has been particularly attractive to the unethical supplier of fats and oils because of marked similarity of these substances and the existence of wide price differentials.

Chemically, most fats and oils are enough alike to make the detection of one oil in the presence of another often quite difficult. If the supplier of edible oils blends oils together in such a way that the final product satisfies the conventional analytical constants used as tests of identity, e.g., refractive index, saponification number, iodine number, etc., adulteration may go undetected. Many specific tests for the detection of one oil in the presence of another depend on non-glyceride constituents. However, modern refining methods that remove these constituents may cause variable results. Because of the large number of possible adulterants, a reasonably complete check on a sample of oil requires the performance of many tests, and hence represents, in most instances, a tedious and time consuming operation. Thus methods of analysis that are rapid, reliable and have a wide application would be very valuable.

A recent problem in this laboratory involved the detection of rapeseed oil in olive oil. Satisfactory chemical tests have been devised (1), but because of the large number of samples involved, a more rapid procedure was sought. While infrared spectroscopy has been widely used for the rapid identification of organic compounds, it has not been used to identify edible oils, since their spectra are very similar. Infrared spectra of a few oils and fats have been reported (2, 3), and the literature in the fats field has been reviewed (4, 5), but no method using infrared spectroscopy to detect one oil in the presence of another has been published. However, in this laboratory in 1954 Stewart showed differences between fats by differential infrared spectroscopy (13), and a suitable method of using this technique was subsequently developed. In the preliminary report on this method (6), the spectra were recorded by using cell lengths of 1 mm. In this paper most of the spectra were obtained by using 0.5 mm cells that gave much more detail in the 1200 to 1080 cm^{-1} region.

The apparatus used for this investigation was a Perkin-Elmer Model 21 infrared spectrophotometer equipped with matched sodium chloride solution cells of 0.5 and 1.0 mm light path. Hypodermic syringes of 0.5 or 1 ml capacity were used for filling the cells.

Experimental

Preliminary investigation in this laboratory showed that the spectra of rapeseed and olive oils, determined in the usual manner, were similar, as shown in Fig. 1, but that there were minor differences in the curves particularly in the 1200 to 900 cm^{-1} region. In order to emphasize these differences, the technique of differential infrared spectroscopy was tried (7). In this procedure, a sample solution is placed in one beam and a reference sample solution in the other beam of the double beam spectrometer. Under these conditions, the instrument measures only the differences between the two beams and does not give any indication of total absorbance. Thus if the same sample is placed in both beams, a straight line will be obtained, whereas if the samples are different, minor differences in the absorption spectra will be evident as deviations above and below the reference line.

If the absorbance of a sample is high, the recorder, which registers linearly with respect to per cent transmittance, records only small changes with changes of 0.1 or 0.2 absorbance units on the chart. However, by

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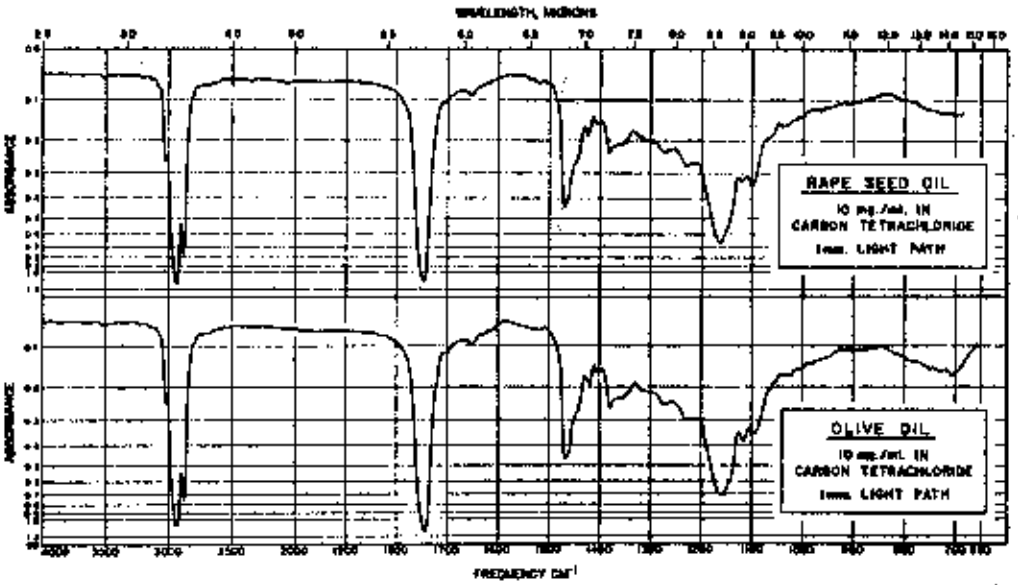

Fig. 1. Infrared spectra of rapeseed oil and olive oil.

using the differential technique, the recording is moved up the chart (to approximately 75% transmission) where the absorbance scale is expanded, and differences of 0.05 absorbance units become apparent. Under these conditions, solutions of high absorbance may be used. Since this technique is essentially the measurement of small differences between two large quantities, we are imposing severe conditions on the apparatus. The cells must be matched as carefully as possible, and the optics of the instrument must be carefully aligned. The amplifier must be stable and free of drift. Even with these precautions, the reference line (i.e., with the same sample in each beam) may show slight deviations from linearity.

Because of the high absorbance of the sample solutions, the recorded differences obtained were not proportional to cell length or concentration since the instrument was unable to record correctly with such low levels of energy. Figures 2, 3, and 4 are differential spectra that illustrate these points. For each curve, a carbon tetrachloride solution of the reference sample of olive oil was placed in the "reference beam" and a solution of a sample consisting of 40% rapeseed oil in olive oil was placed in the "sample beam." The spectra were recorded over the

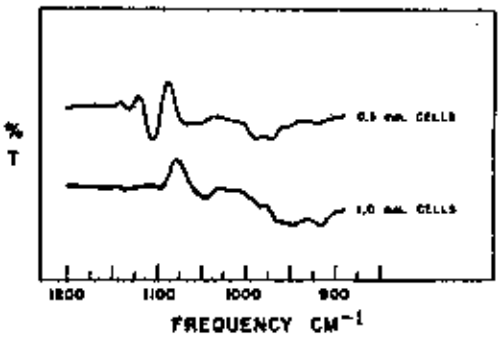

Fig. 2. Effect of cell length on differential spectra of oils.

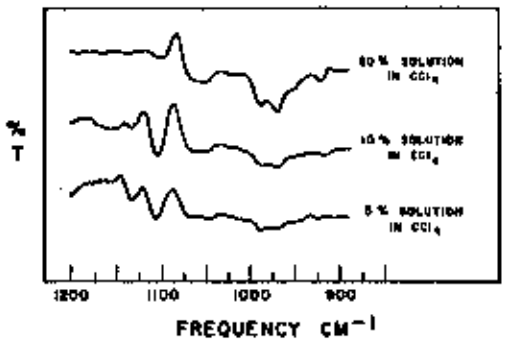

Fig. 3. Effect of concentration on differential spectra of oils.

range 1200–900 cm⁻¹, and except where indicated, 0.5 mm cells, 10% fat in carbon tetrachloride, and a fixed slit of 300μ were used. The beam trimmer balance was set to give a reading of approximately 75% transmission. The effect of cell length is shown in Fig. 2 for 0.5 and 1 mm cells. The region from 1200 to 1075 cm⁻¹ is of such high absorbance that the instrument is "dead" when 1 mm cells are used. A carbon tetrachloride band at about 1000 cm⁻¹ causes

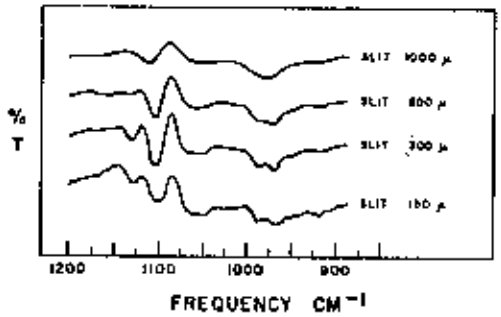

Fig. 4. Effect of slit width on differential spectra of oils.

another "dead" spot with the longer cells. The effect of concentration of oil in the carbon tetrachloride solution is shown in Fig. 3. There is a gain in the differential spectra between 5 and 10% but it is not proportional to the concentration. When 20% solutions are used, the instrument shows little response. The effect of varying the slit is shown in Fig. 4. If an attempt is made to increase resolution with narrower slits the instrument response is poor. When the slits are opened the resolution is reduced and stray light becomes a factor.

In order to use the differential technique, the concentrations of all the samples must be exactly the same so that any differences in absorbance will be due to differences in the spectra, and not reflect differences in concentration of the two samples in the beams. For this reason, oil solutions were prepared accurately on a weight-volume basis. The solvent used was carbon tetrachloride rather than the more volatile carbon disulfide, frequently the solvent of choice. On the basis of these experiments the following procedure was developed.

Procedure

Weigh 2.50 g of an oil sample into a 25 ml volumetric flask and dilute to volume with CCl_4. Prepare a similar 10% w/v solution of olive oil of known purity for reference. Fill two matched 0.5 mm NaCl cells with the reference oil solution and place them in a double beam infrared spectrometer. The following instrumental conditions are used for the Perkin-Elmer Model 21:

Slit	300 μ (fixed)
Response	1:1
Gain	4.8 to 5.2
Filter	out
Frequency range	1200 to 900 cm⁻¹
Prism	sodium chloride
Source current	0.3 amp.
Beam trimmer balance	To give a reading of approximately 75% transmission.

Obtain a tracing which will be a measure of the alignment of the instrument and the matching of the cells. This is referred to as the "reference line." Rinse and fill the sample-beam cell with the solution of the oil under examination, and repeat the recording over the same wavelength region without changing instrumental settings or chart paper.

It was found convenient to use pieces of graph paper 8½×11 inches taped to the recorder drum. Pens with different colored inks for the two tracings were of advantage.

Results

The differential spectra that are presented in Figs. 5 to 21 were obtained by using a 10% w/v solution of a Spanish olive oil in the reference beam of the spectrophotometer. The bottom curve of Fig. 7 shows the tracing obtained with this oil in both beams

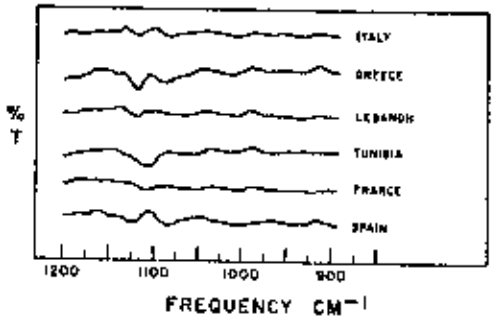

Fig. 5. Differential spectra of olive oils from various sources.

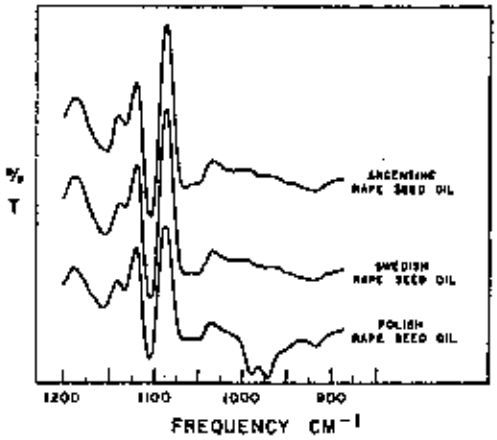

Fig. 6. Differential spectra of rapeseed oil.

and is the curve referred to as the "reference line" in the procedure. In these figures the per cent transmission scale has been displaced vertically for each curve in order to display more than one spectrum in each figure. In most cases the curves would coincide at 890 cm^{-1}. In some spectra the deviations were sufficient that the pen was driven to scale limit, as indicated by flat tops to some peaks. Peaks upward may be considered as negative peaks since they represent regions in which the absorbance is less than that of the reference, while peaks downward are considered as positive.

(*a*) *Differential Spectra of Olive Oil.*—Minor deviations, particularly in the neighborhood of 1100 and 1050 cm^{-1}, were encountered with olive oils from different sources. This procedure has been used to check 252 samples of olive oil from 12 sources. Representative spectra are shown in Fig. 5. The deviations from the reference line at 1125 to 1100 cm^{-1} appear to be due to the natural range of concentration of unsaturated fatty acids encountered in olive oil.

(*b*) *Differential Spectra of Rapeseed Oils and Olive Oil-Rapeseed Oil Mixtures, with Olive Oil as Reference.*—Since adulteration of olive oil with rapeseed oil was suspected, differential spectra of several different types of rapeseed oil and rapeseed-olive oil mixtures were recorded over the range 4000 to 850 cm^{-1}. Differences between the oils were noted at 3100, 1750, and 1400–1300 cm^{-1}, but the most striking features of the differential spectrum were negative peaks at 1130 and 1080 cm^{-1} with a characteristic contour from 1200 to 900 cm^{-1}, which persisted in mixtures containing as little as 10% rapeseed oil. Figure 6 shows the curves obtained from different types of rapeseed oil containing from 25 to 45% erucic acid. The effect of

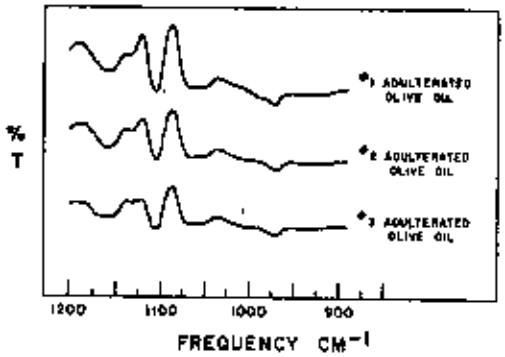

Fig. 8. Differential spectra of olive oil samples adulterated with rapeseed oil.

the addition of rapeseed oil on the iodine number, refractive index, and saponification number of a typical olive oil is given in Table 1. The indicated values give the level of adulteration necessary to bring each of these constants significantly outside the range allowed for olive oil in the Canadian Food and Drug Regulations. Figure 7, which presents the spectra of such mixtures, shows that the infrared procedure is a more sensitive test for adulteration with rapeseed oil since as little as 10% may be detected. Differential spectra of a number of olive oil samples from one supplier showed the characteristics of rapeseed oil adulteration to the

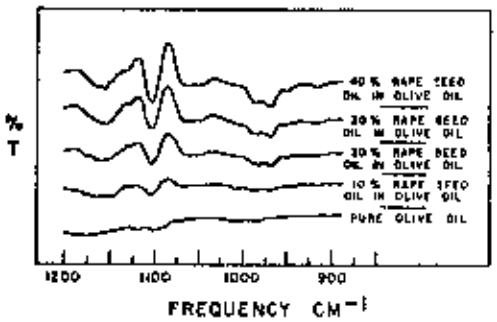

Fig. 7. Differential spectra of rapeseed-olive oil mixtures.

Table 1. Typical analyses of olive-rapeseed oil mixtures

Olive Oil, %	Rapeseed Oil, %	Refractive Index, 30°C	Saponification Number	Iodine Number
100	0	1.4691	189.8	84.8
90	10	1.4696	189.3	88.0
80	20	1.4702	188.1	89.8
70	30	1.4706*	186.3	92.5
60	40	1.4710*	185.3	95.3*
50	50	1.4714*	183.7*	97.8*
Can. F. & D. Limits		1.468–1.470	185–195	77–94

* Values significantly outside the limits of the Canadian Food and Drug Regulations.

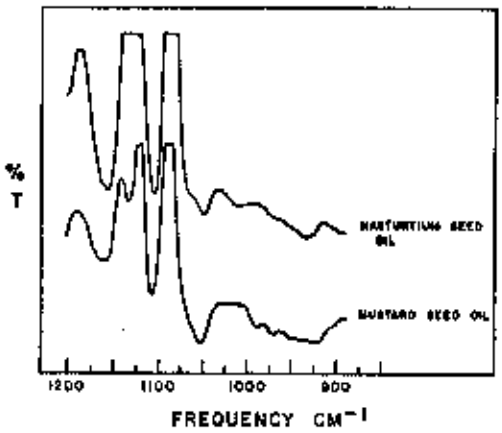

Fig. 9. Differential spectra of oils containing erucic acid.

extent of 25 to 60%. Figure 8 shows the spectra of three of these samples; their analytical values are listed in Table 2.

Nasturtium seed and mustard seed oils, which resemble rapeseed oil in that erucic acid is a major constituent, give differential spectra similar to but not identical with that of rapeseed oil, as shown in Fig. 9.

Table 2. Refractive index, saponification number, and iodine number of commercial olive oil samples adulterated with rapeseed oil

Sample	Refractive Index, 30° C	Saponification Number	Iodine Number
1	1.4709*	185.7	95.7*
2	1.4704*	186.8	91.6
3	1.4702	188.4	93.6
Canadian F & D Limits	1.468–1.470	185–195	77–94

* Values significantly outside limits of Canadian Food and Drug Regulations.

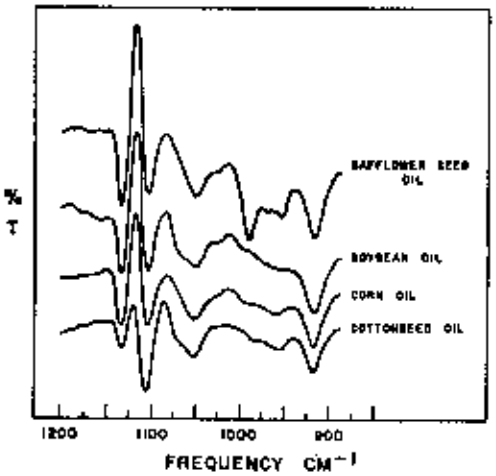

Fig. 10. Differential spectra of vegetable oils.

(c) *Differential Spectra of Vegetable Oils with Olive Oil as a Reference.*—In addition to rapeseed oil, about 30 other vegetable oil spectra have been obtained. The differential spectra with reference to olive oil of some commercially important oils are shown in Figs. 10 to 12 and 20% mixtures of some of these oils with olive oil in Figs. 13 and 14. In addition to the differential spectra presented here, the spectra of a number of other oils have been obtained over the region of 1200 to 850 cm^{-1} in order to permit assignment of spectral characteristics to known constituents. These spectra are not illustrated but the oils studied are listed in Table 3.

(d) *Differential Spectra of Animal Fats and Oils with Olive Oil as a Reference.*—While it is not anticipated that animal fats will be used as adulterants for olive oil, the differential spectra of a number of fats of this type have also been obtained in order to complete the collection of spectra for identification of fats and oils by differential spectroscopy. Six of these spectra are shown in Figures 15 and 16; other fats also tested are listed in Table 3.

Table 3. Oils and fats studied by the differential infrared spectroscopy procedure

Sample	Oil or Fat	No. of Samples	Figure
	A: Vegetable Fats		
1	bayberry[a]	1	
2	boxwood	1	
3	castor	1	
4	coconut	2	11, 21
5	corn	2	10, 14
6	cotton seed	2	10, 14
7	cyperus	1	
8	delphinium seed[a]	1	
9	ergot	1	
10	grapeseed	1	
11	gymnocladus	1	
12	jojoba[b]	1	
13	kenaf	1	
14	linseed	3	
15	mustard seed	1	9
16	nasturtium seed	1	9
17	okra	1	
18	olive	252	1, 5, 17
19	palm	2	11
20	palm kernel	2	11
21	peanut	1	12
22	rapeseed	6	1, 6, 7, 8, 21
23	rice bran	1	
24	safflower seed	2	10, 13
25	sesame	3	12, 14
26	soybean	4	10, 13, 18
27	sunflower seed	1	12, 13
28	tea seed	1	12
29	tung	1	
	B: Animal Fats		
30	butter	29	15
31	herring	3	16
32	lard	4	15
33	menhaden	1	16
34	muskox	1	
35	polar bear	1	
36	rat kidney	5	
37	seal	3	16
38	sperm whale[b]	2	
39	tallow	3	15
40	whale	1	
	C: Hydrogenated Fats		
41	coconut	1	
42	cotton seed	1	20
43	herring	2	
44	margarine	5	
45	palm	1	20
46	palm kernel	1	
47	soybean	4	19
48	seal	3	19

[a] Contained significant amounts of free fatty acids.
[b] Fatty alcohol esters—waxes, not glyceride fats.

(c) The Effect of Refining and Hydrogenation of Oils on Differential Spectra with Olive Oil as Reference.—A number of samples in different states of refinement and hydrogenation were also examined. Refinement of olive oil causes little change in the spectrum

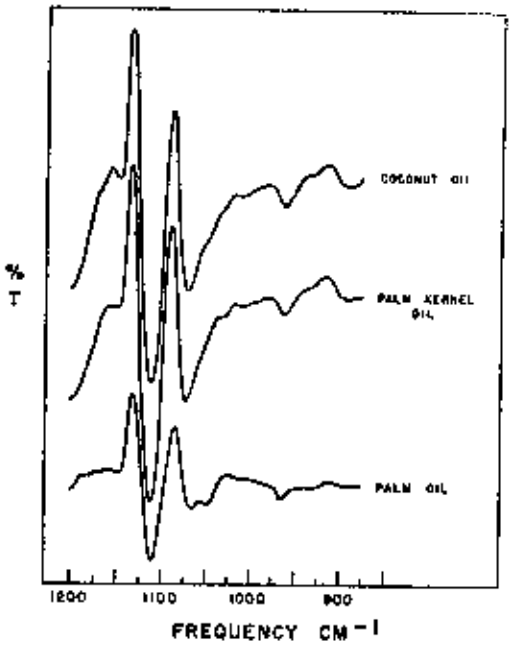

Fig. 11. Differential spectra of vegetable oils.

obtained, as illustrated by Fig. 17. However, with soybean oil, the positive peak at 1050 cm^{-1} encountered with the unrefined oil is noticeably reduced by refinement, as shown in Fig. 18.

The effect of hydrogenation was studied with several types of oil. In all cases the positive peak at 912 cm^{-1} was eliminated and the positive peak at 970 cm^{-1} due to a trans double bond was increased, as shown

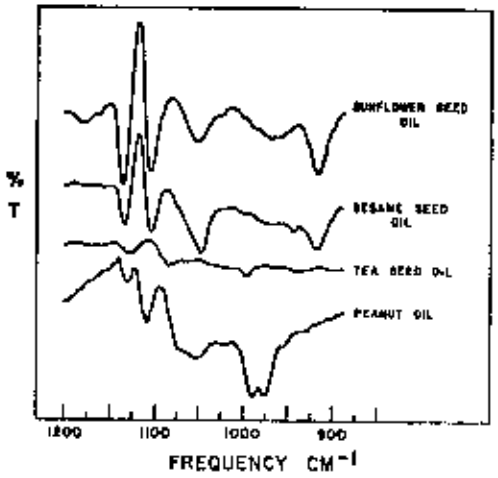

Fig. 12. Differential spectra of vegetable oils.

in Fig. 19. The negative peak at 1100 and 1080 cm^{-1} was gradually increased as hydrogenation became more complete. With fully hydrogenated cotton seed oil and hydrogenated palm oil, the trans double bond peak at 970 cm^{-1} has virtually disappeared, as shown in Fig. 20.

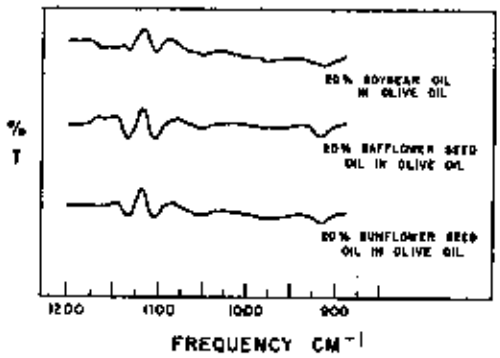

Fig. 13. Differential spectra of vegetable oil-olive oil mixtures.

(*f*) *Differential Spectra of Rapeseed-Coconut-Olive Oil Mixtures with Olive Oil as Reference.*—A mixture containing rapeseed and coconut oils in the proportion of 4:1 has an iodine number, saponification number, and refractive index close to the range of pure

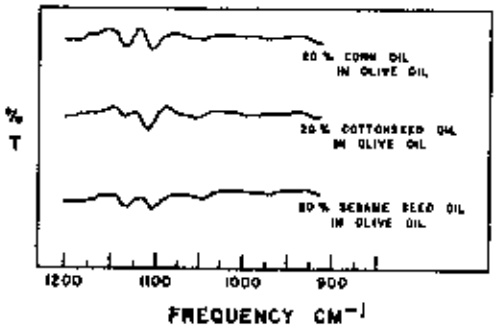

Fig. 14. Differential spectra of vegetable oil-olive oil mixtures.

olive oil. Thus, if this mixture were added to olive oil, the use of these constants would not detect this adulteration. However, the presence of such a mixture is detected spectrophotometrically even at the 10% level of adulteration. The differential spectrum for a 20% mixture is shown in Fig. 21.

Discussion

(*a*) *Applications of Differential Infrared Spectroscopy to Fats and Oils.*—The time required to obtain the differential spectrum of an oil sample is about 10 minutes. For this reason, the procedure is one of the fastest techniques available for checking the purity of an oil sample. Since about 10% to 20% of most fats and oils can be detected in olive oil by this one test, considerable effort can be saved by the elimination of the many tests now necessary to check olive oil for each possible adulterant. Of the oils tested, only tea seed oil showed a spectrum which

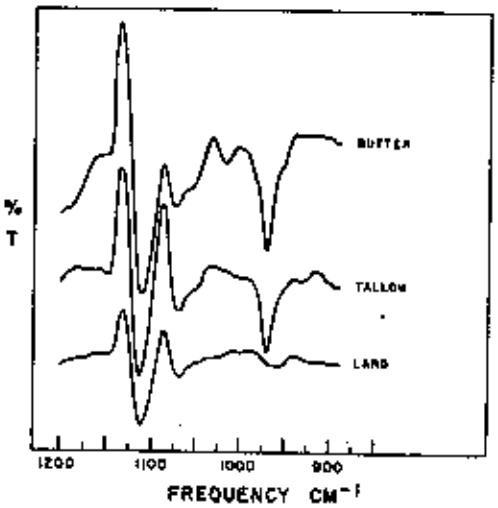

Fig. 15. Differential spectra of animal fats.

was similar to olive oil and would not be detected by this procedure. However, there is a specific color test for tea seed oil (8). An extension of this technique to other oils should be possible for the detection of adulteration. Preliminary tests have shown promise for the detection of lard, tallow, and hydrogenated fats in butter fat.

Since each of the oils tested has a characteristic spectrum, it will be possible to make a rapid identification of many unknown oil samples. Oils that are similar to each other in character, such as soybean, corn, and cottonseed oils, have differential spectra with respect to olive oil that are very similar. However, if one of these is used for reference, differences between these oils become

apparent. Thus, an unknown fat or oil may easily be identified, at least within a group, by differential spectroscopy with olive oil as reference. More positive identification can then be made by obtaining the spectrum of the unknown with one of this group as reference.

Preliminary tests have shown differences in rat kidney fat due to different dietary sources of fat. Kidney fat from rats fed on corn or rapeseed oils showed deviations from that of rats fed on olive oil. These deviations were similar to the differences in the original oils.

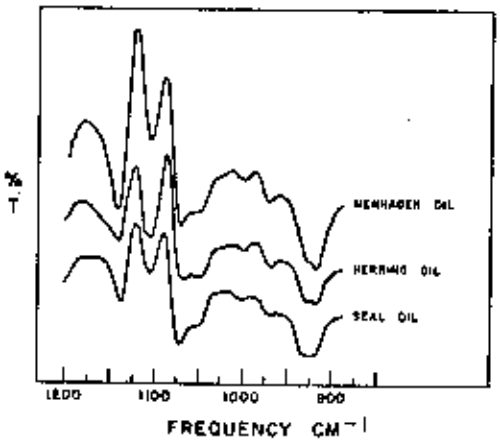

Fig. 16. Differential spectra of marine animal oils.

This technique has also been applied to essential oils and will be the subject of another report (9). The possibility of extending this procedure to flavor extracts is being examined.

(*b*) *Assignment of Spectral Bands.*—It is not the purpose of this report to correlate the differential spectra with specific vibrations in the oil molecule. However, an attempt is made to relate the spectra to the composition of the individual oils.

Prominent differences in the spectra of many of the fats and oils tested occur between 1130 and 1070 cm^{-1}. In the normal spectrum there is a peak at 1112 cm^{-1} (9.0 μ). With relatively saturated fats such as palm kernel and coconut oils, the absorbance is highest and the peak is sharpest. When a fat contains a large proportion of unsaturated acids, this absorbance decreases and the peak broadens or may split into two minor peaks at about 1120 cm^{-1} and 1108 cm^{-1}. A good correlation was obtained between the total of acids unsaturated at the

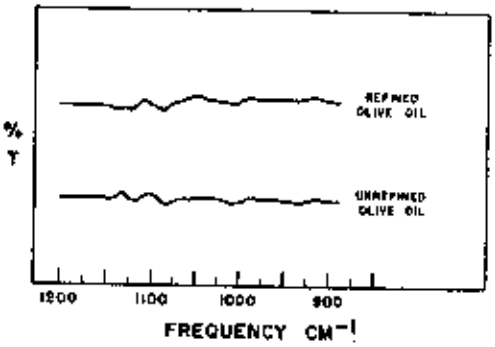

Fig. 17. Effect of refining on differential spectrum of olive oil.

C_9 position and the difference in peak heights at 1112 and 1130 cm^{-1}, by taking measurements from the differential spectra with respect to olive oil, and making correc-

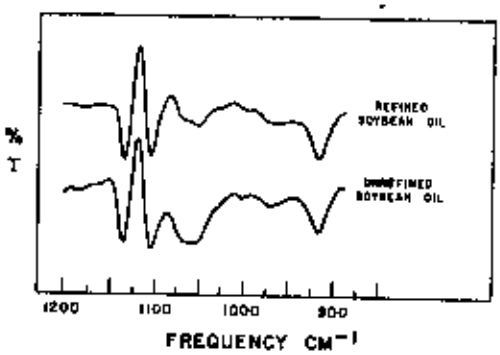

Fig. 18. Effect of refining on differential spectrum of soybean oil.

tions for the molar concentration of the fats. However, it was apparent from the results that linoleic and linolenic acids in fats had about 1.5 times the influence of oleic acid on the peak at 1112 cm^{-1} and that erucic acid (in nasturtium seed, rapeseed, and mustard seed oils) with a double bond at C_{13} has only about 0.5 times the influence of oleic acid. Using these factors, the difference in peak height at 1130 and 1112 cm^{-1} was plotted

against % oleic+1.5×% linoleic+1.5×% linolenic+0.5×% erucic acids, as illustrated in Fig. 22. In this figure the values for the composition of the oils are taken from the literature and do not necessarily represent the exact composition of the oil samples used. The points are identified by number corresponding to the list of oils in Table 3. The effect of chain unsaturation on the spectrum in this region is clearly illustrated in this figure, since the oils represented vary from the saturated type of coconut and palm kernel oils to the highly unsaturated oils such as safflower seed oil.

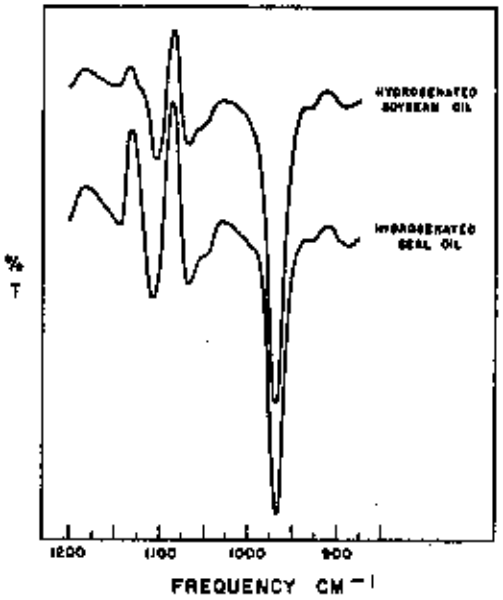

Fig. 19. Differential spectra of partially hydrogenated oils.

Apparently this effect also extends to 1080 cm^{-1}, but the changes are more difficult to analyze, because this region is on the slope of a peak in the normal spectrum. The negative peak observed at 1080 cm^{-1} with rapeseed, coconut oils, etc., in the differential spectra, may be due to a hidden peak in the normal spectrum, or, alternatively, to a shift in peak wavelength. This peak occurs with saturated fats, hydrogenated fats, and, in particular, with oils containing erucic acid (rapeseed oil) or other long chain acids (fish oils).

Fats with short chain-length acids or a low iodine number show a positive peak at about 1065 cm^{-1} that is lacking in oils with significant unsaturation.

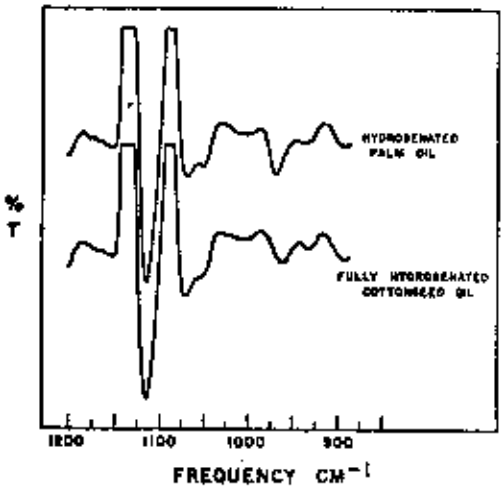

Fig. 20. Differential spectra of hydrogenated fats with low iodine number.

Some oils exhibit a positive peak at 1050 cm^{-1} that has been assigned to the hydroxyl group (2). This peak is present most strongly in the spectra of castor and ergot oils (not illustrated), which contain ricinoleic acid. In some cases, it may also be due to hydroxyl

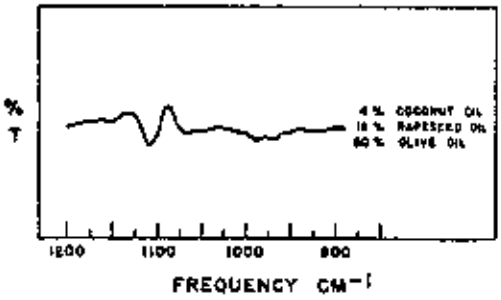

Fig. 21. Differential spectrum of olive-rapeseed-coconut oil mixture.

compounds such as sterols that are minor constituents of the oils. There is a significant decrease in this peak in refined soybean oil as compared to the crude oil (Fig. 18). A cholesterol spectrum, obtained by normal recording, had a strong absorption peak at

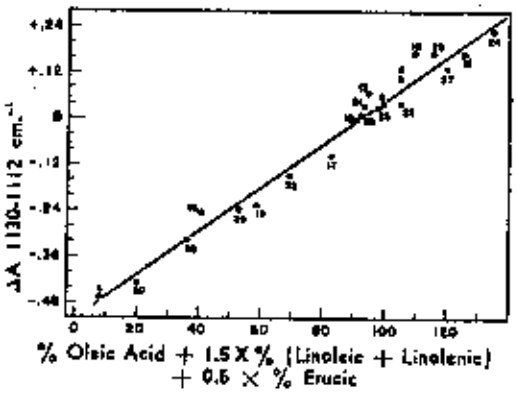

Fig. 22. Effect of unsaturation on differential infrared spectra with respect to olive oil at 1130 and 1112 cm.$^{-1}$

this wavelength. Some olive oils, which exhibited a slight peak at this wavelength, showed no peak upon drying with silica gel, indicating that traces of water may also be responsible for this peak in some samples.

A positive peak at 990 cm^{-1} is present in the spectra of tung oil, safflower seed oil, and some other unsaturated oils. Apparently, it is also related to unsaturation in the fatty acid chain.

The positive peak at 970 cm^{-1} in the spectra of hydrogenated oils and butter has been assigned to the trans-substituted ethylene structure (10).

Oils with a higher iodine value than olive oil show a fairly strong positive peak at 912 cm^{-1}. This appears to be due to multiple unsaturation of the fatty acid chain. This peak is visible in spectra, published by Sinclair, *et al.* (11), of the methyl esters of linoleic, linolenic, and arachidonic acids, but is not present in the spectra of methyl oleate or elaidate.

The spectra of some unrefined oils show a broad positive peak at 940 cm^{-1} that is attributed to the carboxylic group of free fatty acids (12). Delphinium seed oil, which contained about 50% free fatty acid, had a very high absorbance at this frequency.

Acknowledgments

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The use of fats in pig feeding

II. The digestibility of various fats and fatty acids

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The efficient formulation of diets for farm animals demands a knowledge of the relative merits in terms of nutrient supply of the different feed ingredients that are available. In this respect it can be stated that supplementary fats are added as sources of energy and their relative values must therefore be assessed in units of digestible energy (see Robinson, Lewis & Morgan, 1964). Renner & Hill (1960) determined the metabolizable energy values of several fats using the chick, by substituting fats for carbohydrate in the diet and evaluating the gross energy of combustion of feed and excreta under carefully standardized conditions. They also compared such a value with one obtained as a product of the gross energy of combustion of the fat and its corrected digestibility. The former procedure unfortunately allocates to the fat component any potentially variable change in utilization of the other dietary components brought about by the presence of the fat. The second procedure refers only to the fat fraction but does demand an assessment of corrected digestibilities, which in turn requires an evaluation of faecal fat not of immediate dietary origin (Bayley & Lewis, 1965). In the present studies the digestible energy value was calculated from corrected digestibility; attempts at a direct determination were discarded partly because of the difficulties of obtaining fully representative small samples of feed and faeces for use in the bomb calorimeter.

Triglycerides and free fatty acids, constitute the bulk of fats likely to be added as supplements to pig feeds. The fatty acid composition of the mixtures varies considerably and it is fully acknowledged that the degree of absorption of the fat depends upon the fatty acids present (Renner & Hill, 1958). In the present studies the digestibility of each of the component fatty acids of the fat has been determined separately in an attempt to show the extent to which the absorption of each fatty acid is influenced by the others present in the diet. Three commercially available fats were examined: beef tallow, H.E.F. (a partially hydrolysed mixture of plant and animal fats supplied by Procter and Gamble, Newcastle-upon-Tyne) and degummed soyabean oil. In addition to these fats a group of other materials was examined; oleic, stearic and palmitic acids both as free fatty acids and specific triglycerides.

EXPERIMENTAL

The detailed experimental procedure was described by Bayley & Lewis (1965). The fats were added (at 5 %) to the basal diet described in Table 1. The composition of the rations containing the commercially available fats is also shown in Table 1. The composition of the rations containing the semi-purified fats are shown in Table 2. The rations were prepared at different times and thus the small differences in their compositions shown by the analysis could be the result of differences in the composition of the basal ingredients.

The total faecal fat and fatty acid output were arbitrarily divided into that component which represented unabsorbed dietary fat (exogenous faecal fat) and that which was not directly derived from the dietary fat (endogenous faecal fat). This was accomplished by assuming that the production of the latter is unaffected by the amount of fat in the diet and that the faecal fat output on a fat free diet represents the fat which is of endogenous origin. The values determined by Bayley & Lewis (1965) were used in these studies to calculate corrected digestibilities.

The pigs were given 2 lb./day of the experimental rations with the exception of the ration containing the H.E.F. These pigs weighed 60 lb. at the beginning of the experiment and they were given 2½ lb. of meal per day. In the experiments with the semi-purified fats, two of the pigs were given the rations containing the free fatty acids and two the rations containing the triglycerides.

RESULTS

The food and faecal lipids were extracted and analysed into their component fatty acids and these results are shown in Table 3. The basal ingredient of the rations contributed 2 % of fat and this was present in all diets. Thus the dietary lipid is

Table 1. *Rations used in the determination of the digestibility of some commercially available fats*

	Composition (%)			
Weatings (5 % fibre)	10	10	10	10
Barley meal	72	66	66	66
Extracted soyabean meal	10	10	10	10
Extracted white fish meal (Provimi '66')	5	6	6	6
Vitamin mineral supplement*	3	3	3	3
Beef tallow	—	5	—	—
H.E.F.	—	—	5	—
Soyabean oil (degummed)	—	—	—	5
	Results of analysis (as % of dry matter)			
Dry matter in ration (as % of original ration)	86	86	86	85
Crude fibre	4·8	4·9	4·9	3·1
Ether extract	1·9	6·5	6·6	7·9
Acid ether extract	0·7	0·7	0·7	0·7
Crude protein (% N × 6·25)	19·3	19·3	18·3	20·3
Ash	4·8	4·8	4·8	4·6
Calcium	0·7	0·8	0·8	0·8

* The supplement supplied the following levels of vitamins and minerals to the final ration: calcium, 0·8 %; phosphorus, 0·4 %; common salt, 0·35 %; iron, 45 p.p.m.; manganese, 20 p.p.m.; zinc, 100 p.p.m.; iodine, 0·5 p.p.m.; vitamin A, 1000 i.u./lb.; vitamin D_3, 250 i.u./lb.; pantothenic acid, 5 mg./lb.; nicotinic acid, 5 mg./lb.; riboflavin, 1·5 mg./lb.

Table 2. *Analysis of rations containing the semi-purified triglycerides and free fatty acids*

Results expressed as percentage of dry matter. Triglyceride or fatty acid added (5 %) to the ration is indicated above each column.

	Triolein	Oleic acid	Tristearin	Stearic acid	Tripalmitin	Palmitic acid
Dry matter (as % of original ration)	90·0	89·4	89·1	89·5	87·2	86·5
Crude fibre	2·0	1·7	1·8	1·9	3·3	3·3
Ether extract	7·0	7·0	6·6	7·0	7·5	7·5
Acid ether extract	0·4	0·3	0·4	0·4	0·5	0·5
Crude protein (% N × 6·25)	17·6	18·1	18·6	18·8	19·2	19·0
Ash	6·3	6·6	6·6	6·5	6·9	6·5
Calcium	0·7	0·8	0·8	0·7	0·7	0·8

Table 3. *Fatty acid composition of basal ration and the rations containing beef tallow, H.E.F. and soyabean oil*

The fatty acid composition of the faecal lipids produced by the pigs receiving these rations are also indicated.

	Percentages							
	Basal ration		Ration with added beef tallow		Ration with added H.E.F.		Ration with added soyabean oil	
	Food	Faeces	Food	Faeces	Food	Faeces	Food	Faeces
Acids								
14:0	0·8	1·2	3·5	2·1	3·3	2·1	0·2	1·1
15:0	1·7	5·3	0·8	3·0	0·3	3·6	0·1	4·5
16:1	0·8	0·2	0·2	0·5	0·9	0	0	0
16:0	38·2	25·2	27·0	33·2	29·2	30·7	17·1	23·8
17:0	0	2·8	1·4	1·5	1·2	0	0	2·6
18:2	32·6	2·0	13·6	1·5	15·6	0·9	63·3	3·2
18:1	15·7	12·2	36·2	8·0	33·1	8·2	15·8	13·8
18:0	10·2	50·8	16·2	49·8	16·2	54·8	3·6	52·0

constituted of this plus the added fat. The composition of the dietary fat covers a wide range, from the beef tallow and H.E.F. which have a high saturated fatty acid content to the soyabean oil with a low level of saturated fatty acid. The fatty acid composition of the faecal lipids is little affected by these alterations in the food lipids. Table 4 shows the corresponding figures for the rations containing the semi-purified fats. These show that the dietary lipids were not composed exclusively of the nominal fatty acid being studied. The faecal lipids produced by the pigs receiving the diets containing linoleic and oleic acid had a similar fatty acid composition to the faecal lipids produced by the pigs receiving the commercial fats. However, the addition of palmitic acid, stearic acid or their corresponding triglycerides to the diets increased the contribution of these acids to the faecal lipids.

The results of the balance experiments and of the analysis of the food and faecal lipids allowed the intake and output of each acid to be calculated. The values for the pigs receiving the basal ration and the ration containing the commercial fats are presented in Table 5. The faecal fatty acid output recorded for pigs receiving the extracted basal ration (Bayley & Lewis, 1965) are included in the table for comparison. The output of pentadecanoic, heptadecanoic, linoleic and oleic acids are relatively constant in spite of the variation in fatty acid intakes and are of the same order as those recorded for the extracted basal ration. The outputs of palmitic and stearic acids show wide variations which are to some extent related to the variations in intake. Table 6 shows the corresponding values for the pigs receiving the rations containing the semi-purified fats. The outputs of pentadecanoic, heptadecanoic and linoleic acids are again relatively constant. However, the major component acid of the diets in each case led to an increase in the output of that acid in the faeces.

The apparent and corrected digestibilities of the total dietary lipid and the major component acids are shown in Table 7. The saturated acids are less well absorbed than the unsaturated acids. The corresponding values for the semi-purified fats are shown in Table 8. The values for the total lipid and the determined main fatty acid do not agree because of the other acids present in the dietary lipid. The digestible energy values of the fats in the ration are shown in Table 9. These were determined as the

Table 4. *Fatty acid composition of rations containing the semi-purified triglycerides and free fatty acids and the fatty acid composition of the faecal lipids produced by the pigs receiving these rations*

	Triolein		Oleic acid		Tristearin		Stearic acid		Tripalmitin		Palmitic acid	
	Food	Faeces	Food	Faeces	Food	Food	Food	Faeces	Food	Faeces	Food	Faeces
Acids												
14:0	3·3	1·7	4·6	1·2	0·2	0·1	1·4	0·3	2·9	1·9	2·6	1·6
15:	0	5·9	0	6·6	0	1·1	0	1·1	1·3	2·0	1·1	1·7
16:1	5·7	0·5	6·7	0·9	0	0	0	0	0·7	0·3	0·9	0·4
16:0	24·7	25·3	16·1	20·5	18·2	14·0	16·3	13·0	68·8	69·5	74·3	70·8
17:	0	4·5	0	4·6	0	1·5	0	0·8	2·5	3·3	2·6	2·0
18:2	0	3·4	0	3·0	0	0·7	0	0·8	1·0	2·2	0·9	1·4
18:1	77·0	11·7	71·2	19·8	12·7	20·2	11·7	9·7	17·4	5·2	12·8	3·5
18:0	8·0	47·0	1·2	43·5	60·0	62·0	72·0	74·1	5·3	15·4	5·1	11·4

Table 5. *Total fat and individual fatty acid balance for pigs receiving basal ration and rations containing beef tallow, H.E.F. and soyabean oil (g/day)*

	Extracted basal ration, output	Basal ration		Beef tallow ration		H.E.F. ration		Soyabean oil ration	
		Intake	Output	Intake	Output	Intake	Output	Intake	Output
Total lipid ...	11·5	19·8	13·6	55·6	19·6	70·0	28·4	66·3	15·7
Saponifiable ... lipid	9·6	10·8	12·2	63·1	18·8	66·8	25·7	63·6	14·3
Acids									
14:0	0·2	0·1	0	1·9	0·4	2·2	0·6	0·1	0·2
15:	0·7	0·3	0·7	0·4	0·6	0·2	1·0	0·1	0·7
16:1	0·1	0·1	0	0·1	0·1	0·6	0	0	0
16:0	2·3	6·4	3·1	14·4	6·3	19·4	8·0	10·9	3·4
17:	0·4	0	0·3	0·7	0·3	0·8	0	0	0·4
18:2	0·2	5·6	0·2	7·5	0·3	10·4	0·2	40·3	0·3
18:1	1·0	2·6	1·5	19·2	1·7	22·4	2·3	10·0	2·0
18:0	4·7	1·7	6·3	8·6	9·4	10·8	14·0	2·3	7·5

Table 6. *Total fat and fatty acid balances for pigs receiving the rations containing the semi-purified triglycerides and free fatty acids (g./day)*

	Triolein		Oleic acid		Tristearin		Stearic acid		Tripalmitin		Palmitic acid		Extracted basal ration, output
	Intake	Output	Intake	Output	Intake	Output	Intake	Output	Intake	Output	Intake	Output	
Total lipid ...	60·4	15·9	59·8	12·6	55·4	26·5	59·5	35·3	62·9	25·5	62·6	44·4	11·5
Saponifiable lipid ...	58·3	14·2	58·2	11·1	52·2	34·9	54·6	34·2	59·7	23·4	59·5	41·7	9·6
Acids													
14:0	1·9	0·2	2·6	0·1	0·1	0	0·8	0	1·7	0·5	1·6	0·7	0·2
15:0	0	0·8	0	0·7	0	0·4	0	0·4	0·8	0·5	0·6	0·7	0·7
16:1	3·3	0·1	3·9	0·1	0	0	0	0	0·4	0·1	0·4	0·2	0·1
16:0	14·0	3·6	9·4	2·3	9·5	5·2	6·3	4·4	40·8	16·2	44·2	22·0	2·3
17:0	0	0·7	0	0·5	0	0·5	0	0·3	1·5	0·3	1·5	1·2	0·4
18:2	0	0·5	0	0·3	0	0·2	0	0·3	0·9	0·5	0·6	0·6	0·2
18:1	33·8	1·7	41·5	2·2	6·6	7·1	6·4	3·3	10·3	1·2	7·6	1·5	1·0
18:0	4·7	6·7	0·7	4·8	36·2	21·6	39·2	25·3	3·1	3·6	3·0	4·8	4·7

product of gross energy value and corrected digestibilities. Since the gross energy value did not vary greatly from sample to sample the differences essentially reflect the difference in digestibility.

DISCUSSION

There is little quantitative information available upon the digestibility of fat in the pig. The apparent digestibility of the ether extract fraction of rations containing added fats has been determined by a number of authors. Clawson, Blumer, Smart & Barrick (1962) found values ranging from 47 % for the ether extract of a ration containing no added fat to 75 % for a ration containing 10 % of beef tallow. On the other hand, Lloyd & Crampton (1957) quoted values from 78 to 94 % for diets containing 20 % of a range of added fats. More detailed studies have been carried out with poultry. Renner & Hill (1961*a*) recorded a corrected digestibility of 69 % for beef tallow using 3–4-week-old chicks and one of 96 % for soyabean oil. These values can be compared with the 85 and 94 % respectively found for pigs of 50–60 lb. live weight used in the present experiment.

The values for the absorbability of free palmitic, stearic and oleic acids of −1, −2 and 86 % respectively, reported by Renner & Hill (1961*b*) are lower than the corresponding values observed in the present trials with pigs, namely, 38, 44 and 97 % respectively. However, the fatty acids used by those authors were 90 % pure in contrast to the

Table 7. *Apparent and corrected values for the digestibility of the fat content of the basal ration and of the ration containing beef tallow, H.E.F. and soyabean oil*

	Basal ration	Beef tallow	H.E.F.	Soyabean oil
	Apparent digestibilities (%)			
Total lipid	29	65	60	76
Acids				
14:0	*	78	72	*
16:1	46	55	100	*
16:0	60	55	33	68
18:2	95	96	98	99
18:1	40	91	89	89
18:0	*	*	*	*
	Corrected digestibilities (%)			
Total lipid	87	85	73	94
Acids				
14:0	75	88	80	95
16:1	100	100	100	*
16:0	87	72	68	91
18:2	98	99	99	99
18:1	78	97	94	99
18:0	*	46	*	*

* Denotes that output exceeds intake.

Table 8. *Apparent and corrected values for the digestibilities of the fat content of the rations with the added semi-purified triglycerides and free fatty acids*

The digestibilities of the total lipid and component acids are shown separately.

	Oleic		Stearic		Palmitic	
	Triglyceride	Free acid	Triglyceride	Free acid	Triglyceride	Free acid
	Apparent digestibility (%)					
Total lipid ...	73·6	78·9	34·1	40·7	59·0	29·0
Acids						
16:0	73·2	75·5	41·5	43·5	58·1	27·9
18:1	95·1	94·6	*	49·3	87·8	80·8
18:0	*	*	35·3	31·1	*	*
	Corrected digestibility (%)					
Total lipid ...	92·7	98·8	54·7	60·0	77·0	47·4
Acids						
16:0	90·2	99·2	73·1	72·8	64·1	33·3
18:1	98·3	97·2	0·5	63·0	97·5	93·4
18:0	52·2	45·9	49·1	43·7	100	96·8

* Output exceeded intake.

Table 9. *Digestible energy values of commercial fats (kcal./kg.) calculated as product of gross energy value and corrected digestibility*

	kcal./kg.
Beef tallow	7940
H.E.F.	7010
Soyabean oil	8430

approximately 70% purity of the samples used in the present studies. Carroll (1958) fed rations containing palmitic, stearic and oleic acids to rats and reported the following corrected digestibilities: 48, 12 and 84%. Carroll & Richards (1950) found that tristearin and tripalmitin were less well digested than the corresponding free fatty acids in the rat, but triolein was better digested than oleic acid. This is in agreement with the observations of Mattson (1959) that tristearin has a very low digestibility. The comparatively high values for tristearin found in the present studies, with the pig, 60%, agrees well with the 59% digestibility of mono-oleo-distearin reported by Mattil & Higgins (1945) in the rat. Hoagland & Snider (1943) found that beef tallow had a corrected digestibility of 87% in the rat which is in good agreement with the 85% found in the pig.

These results suggest that although the digestibilities of the individual fatty acids in the pig follow a similar pattern to those in other species with the unsaturated acids being better absorbed than the saturated ones, the absorption of a particular acid is to a large extent influenced by the other acids in the fat. Palmitic acid is 64% digested in the semi-purified tripalmitin, 72 and 68% in the beef tallow and H.E.F. containing diets, 91% in the soyabean oil and 90% in the triolein. The large excess of stearic acid in the faeces as compared to the amount in the food obscures the effects of other acids in the diet on the utilization of the stearic acid. Only in the diets containing tristearin and stearic acid is the digestibility of the oleic acid depressed by a large intake of saturated fatty acid.

The high digestibility of the unsaturated acids in the rations and the low digestibility of the saturated acids indicates that the absorption of the unsaturated acids is largely independent of the absorption of the saturated acids. A complete hydrolysis of the dietary triglycerides would allow this independence in the absorption of the different acids. Hydrolysis of the lipid would be aided by an increase in the degree of dispersion of the oil in the aqueous medium so that a greater surface area of oil would be available for the action of pancreatic lipase which only acts at the oil-water interface (Desnuelle, 1961). Breyer & Goodwin (1962) showed that the interfacial tension in an oil-water emulsion was decreased by an increase in the unsaturation of the oil. This feature would account for the higher digestibility of the palmitic acid in soyabean oil than in beef tallow. The low digestibility of the oleic acid present in the rations containing semi-purified stearic acid and tristearin could be the result of a general failure of dispersion. These results show little difference in the utilization of fat when it is fed in the form of the triglyceride or free fatty acid with the exception of the low utilization of the palmitic acid when this was present in the free state.

The results of these experiments are not wholly in accord with the 'partitioning theory' of absorption put forward by Frazer (1943) but indicate that the absorption process is selective and probably requires an extensive hydrolysis of the dietary triglyceride

SUMMARY

The apparent and corrected digestibilities of beef tallow, H.E.F. and soyabean oil have been determined in the newly weaned pig. Determinations of the individual fatty acids in the food and faecal lipids allowed the digestibility of each acid to be calculated separately. The purest available forms of oleic, stearic and palmitic acids and their corresponding triglycerides were also used in the determination of digestibility. Although the digestibility of each fatty acid is to some extent a characteristic of the acid, the extent of absorption can also be modified by the other acids in the diet. The results of these experiments give no clear indication of a difference between feeding fatty acids in the free form or as the triglycerides. The digestible energy values of the fats have been calculated as the product of gross energy value and corrected digestibility.

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The Use of High Quality Fat and the Effect of Protein Level in Broiler Diets

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INTRODUCTION

RECENT surpluses of animal fats in the U.S.A. have stimulated interest in its wide incorporation into broiler diets. The resulting high energy diets have been shown to improve the efficiency of feed utilization when measured as pounds of feed consumed per pound of gain (Sunde, 1956; Donaldson *et al.*, 1957). This improvement may possibly be due partly to lowered feed wastage than occurs with light, bulky mashes. It has also been shown that high energy

diets, by reducing volume of feed intake will reduce the amount of each nutrient consumed and show up deficiencies of marginal nutrients, unless the nutrient is suitably more concentrated in the diet (Thayer and Davis, 1953; Peterson, Grau and Peak, 1954; Baldini and Rosenberg, 1955).

Although the animal fats at present obtainable in N.S.W. are more expensive per unit of energy than more conventional energy sources in poultry mashes, it is conceivable that a greater supply of animal fat in the future may lead to the reversal of this situation, particularly with lower grade tallows. Experiments were therefore conducted to provide information on the value of tallow in broiler mashes based on crushed wheat and meat meal.

EXPERIMENTAL

Experiment 1. The experimental diets were fed as all mashes and *ad libitum*. Details are given in Table 1.

Three basal diets were mixed. They were characterized as follows:

1. 20% Protein, no vitamin supplements
2. 20% Protein, vitamin B supplement
3. 25% Protein, vitamin B supplement

Basal diets were varied by replacing 5 lbs. of wheat meal with 5 lbs. of unstabilized high quality tallow, or 5 lbs. of high quality tallow stabilized by 0.015% Progallin LA (Nipa Laboratories) and 0.01% citric acid. The stabilizing agents were added to the fat when the sample was received, about 2 months before mixing of the mashes. The fat was stored at room temperature, being frequently in a liquid form in the warm weather.

These diets were fed to 8 weeks of age, to 9 randomized groups of 25 newly hatched White Leghorn × Australorp cross-

TABLE 1.—*Composition of diets for experiment 1 (percent)*

Constituents[1]	Normal			High B vitamin			High B vitamin + High protein		
	A	B	C	D	E	F	G	H	I
Wheat Meal	73	67	67	72½	66½	66½	61½	52½	52½
Meat Meal (50% protein)	9	9½	9½	9	9½	9½	14½	16½	16½
Peanut Meal	9	9½	9½	9	9½	9½	14½	16½	16½
Buttermilk Powder	5	5	5	5	5	5	5	5	5
Liver Meal	3	3	3	3	3	3	3	3	3
Salt	½	½	½	½	½	½	½	½	½
Basal Additions[2]	½	½	½	½	½	½	½	½	½
Fat	—	5	—	—	5	—	—	5	—
Stabilized Fat	—	—	5	—	—	5	—	—	5
B Vitamin Concentrate[3]	—	—	—	½	½	½	½	½	½
Est. Protein Content (%)	20	20	20	20	20	20	25	25	25
Est. Productive Energy (Cal./lb.)	881	980	980	877	975	975	893	970	970

[1] For average composition of feedstuffs see McClymont and McDonald (1958), Scientific Poultry Feeding—5th Ed.

[2] Basal Additions (p.p.m.) made up to ½ percent with pollard:

DL Methionine	1,000
Riboflavin	4.4
Procaine Penicillin	7.3
Manganese Sulphate	110
Vitamin A/D_3 Concentrate (containing 50,000 I.U. vit. A and 5,000 I.C.U. vitamin D_3 per gm.—Hoffman La-Roche)	110

[3] B Vitamin Concentrate (p.p.m.) made up to ½ percent with pollard:

Nicotinic acid	8.8
Calcium Pantothenate	8.8
Thiamine	4.4
Riboflavin	4.4
Pyridoxine	4.4
Folic Acid	2.2
Biotin	0.37

TABLE 2.—*Composition of diets for experiment 2 (percent)*

Constituents (see Table 1)	A	B	C	D	E	F
Wheat Meal	86½	86½	81	81	72	72
Meat Meal (50% protein)	5½	5½	6½	6½	16½	16½
Buttermilk Powder	3	3	3	3	3	3
Liver Meal	3	3	3	3	3	3
Salt	½	½	½	½	½	½
Basal Additions[1]	+	+	+	+	+	+
Fat	—	—	5	5	5	5
Limestone Powder	1½	1½	1	1	—	—
DL Methionine	—	0.1	—	0.1	—	0.1
Est. Protein Content (%)	15	15	15	15	19	19
Est. Productive Energy (Cal./lb.)	879	879	982	982	989	989

[1] Basal Additives (p.p.m.) premixed with wheat meal:

Procaine Penicillin	4.4
Manganese Sulphate	110
Vitamin A/D_3 Concentrate (50,000 I.U. vit. A and 5,000 I.C.U. vit. D_3 per gm. —Hoffman La-Roche)	110

bred cockerel chickens housed in a water heated battery brooder, followed at four weeks of age by wire floored water heated cages. The chickens were vaccinated with pigeon pox vaccine at 7 days and electrically debeaked to remove portion of the top beak, at 3 weeks of age. The individual body weights at 4½ and 8 weeks of age were recorded.

Experiment 2. At 8 weeks of age the cockerels were subjected to a change of diet and carried on to twelve weeks in colony cages. Basal diets with 15% protein and no added fat, 15% protein and 5% fat, and 19% protein and 5% fat were compared with similar diets to which 0.10% methionine was added. The added fat consisted of equal parts of the original unstabilized and stabilized fats. The diets are given in Table 2.

Reallocation of cockerels from experiment 1 was achieved by taking 3 birds at random from each of the original 9 groups to make up one of the groups of experiment 2. Six groups of 27 birds were thus obtained. The surplus birds were discarded from the experiment. Individual body weight and length of shank at 8 and 12 weeks of age were recorded, as well as feed consumption over the period.

RESULTS

Over-all mortality to 8 weeks was 15.6%. A large proportion of these deaths (31/35) occurred after debeaking as a result of faulty technique causing improper cauterization, which allowed bleeding. This was confirmed by post mortem findings of death by hemorrhage. It may be significant that the diets contained no added vitamin K. The other deaths (4/35), which occurred before debeaking were diagnosed as naval infection. These mortalities were not specific to treatments. No deaths occurred between 4½ and 8 weeks and mortality in experiment 2 was very low (0.02%).

a) Body weights—Experiment 1. Table 3 shows the group means of weights of chickens at 4½ weeks of age. Fat had no significant effect on body weight. Lack of vitamin B supplement had no significant effect on body weight, which means that the other constituents of the diet supplied all the B vitamins tested in the supplement, in sufficient quantities. Increasing the protein level of the basal diet to 25% produced marked improvements ($P < 0.01$).

Group means of body weights at 8 weeks of age are summarized in Table 4. Essentially the same pattern as at 4½ weeks was present, with the 25% protein basal diet

TABLE 3.—*Mean weights of chickens at 4½ weeks of age (lbs.)*

Basal diet	Normal			High vit. B			High vit. B High protein		
Added Fat	Nil	5% U.S.	5% S.	Nil	5% U.S.	5% S.	Nil	5% U.S.	5% S.
Mean Wt. of Group	0.503	0.474	0.503	0.482	0.470	0.474	0.692	0.668	0.708
Mean Wt. for Basal Diets		0.493			0.475			0.689	

Least significant difference between basal diets (P=0.05) (0.030)
(P=0.01) (0.040)

U.S.—Unstabilized Fat.
S.—Stabilized Fat.

TABLE 4.—*Mean weights of chickens at 8 weeks of age (lbs.)*

Basal diet	Normal			High vit. B			High vit. B High protein		
Added Fat	Nil	5% U.S.	5% S.	Nil	5% U.S.	5% S.	Nil	5% U.S.	5% S.
Mean Wt. of Group	1.266	1.186	1.268	1.214	1.287	1.215	1.462	1.435	1.555
Mean Wt. for Basal Diets		1.240			1.237			1.464	

Least significant difference between basal diets (P=0.05) 0.063 lb.
(P=0.01) 0.084 lb.

U.S.—Unstabilized Fat.
S.—Stabilized Fat.

producing a significant weight increase ($P < 0.01$), while fat and vitamin B supplementation had no significant effect.

Experiment 2. Group means of increases in body weight between 8 and 12 weeks are shown in Table 5.

High protein level (19%) again showed the greatest effect ($P < 0.01$). The analysis of variance of the increases showed that the effect of methionine supplementation was also significant ($P < 0.05$), and from Table 5 it is seen that the methionine response occurs at the low protein level (15%). The interaction between methionine and basal diets indicated by the group means was not significant.

Body weights at 12 weeks were analysed. Table 6 shows the analysis of variance. This analysis summarizes the effect on 12 weeks body weight of both experiments.

b) Shank length. Shank length at 8 weeks was found to be highly correlated with body weights within groups ($r = +0.998$; $P < 0.001$). Similarly the increase in shank length between 8 and 12 weeks was significantly correlated within groups, with increase in body weights between 8 and 12 weeks ($r = +0.461$; $P < 0.01$). Between treatments significant variation independent of body weight, or body weight increase, occurred in shank length at 8 weeks and in shank length increase between 8 and 12

TABLE 5.—*Means of body weight increases between 8 and 12 weeks (lbs.)*

Basal diets	0 fat, low protein		5% fat, low protein		5% fat, high protein	
0.10% Methionine	Nil	+	Nil	+	Nil	+
Mean Wt. of Group	0.889	1.008	0.966	1.080	1.212	1.209
Mean Wt. for Basal Diets	0.949		1.023		1.211	

Least significant difference between basal diets (P=0.05) 0.089
(P=0.01) 0.117

TABLE 6.—*Analysis of variance of body weight at 12 weeks*

Source	d.f.	S.S.	M.S.	F.
Cells	52	10.6362		
Within Cells	103	7.1574	0.0695	
Treatment 0–8 weeks	8	4.8274		
Fat	2	0.2477	0.1239	1.783
Basal Diets	2	4.5288	2.2644	32.581**
Interaction	4	0.0509	0.0127	0.183
Treatment 8–12 weeks	5	2.0410		
Methionine	1	0.4077	0.4077	5.866*
Basal Diets	2	1.3922	0.6961	10.016**
Interaction	2	0.2411	0.1206	1.735
Interaction (before by after 8 weeks)	39	0.2890	0.0074	0.106

* Significant at P = 0.03.
** Significant at P = 0.01.

weeks respectively. The correlation coefficients ($r = +0.292$ and $r = +0.354$ respectively) were not significant.

Analysis of shank length at 8 weeks showed a significant increase ($P < 0.01$) in length due to high protein in the basal diet. Table 7 gives the mean shank lengths at 8 and at 12 weeks.

Aanalysis of shank length increase between 8 and 12 weeks showed no significant effects due to basal diets or methionine but a negative interaction between basal diets and methionine ($P < 0.01$). Methionine responses are shown in Table 8.

TABLE 8.—*Increase in shank length between 8 and 12 weeks (mm.)*

Basal diet	No. methionine	With methionine	Response
No fat, 15% protein	21.3	23.2	+2.0
5% fat, 15% protein	21.7	22.5	+0.8
5% fat, 19% protein	24.7	20.9	−3.8

Analysis of 12 weeks shank length as well as showing the above two effects highly significant ($P < 0.01$), shows a significant effect ($P < 0.05$) caused by fat in the treatments from 0 to 8 weeks. Table 7 shows that the mean shank length at 12 weeks, of birds receiving unstabilized fat was significantly shorter than that of birds receiving no fat in the diet. The group receiving stabilized fat was intermediate (difference no significant).

c) Feed Conversion. Experiment 1—Feed consumption data to 8 weeks are summarized in Table 9. Up to 4½ weeks results are somewhat confused by the heavy mortalities following debeaking. No deaths occurred between 4½ and 8 weeks, so that feed conversion (feed consumed/pound weight increase) could be accurately de-

TABLE 7.—*Mean shank lengths at 8 and 12 weeks (mm.)*

Basal diet	Normal			High vit. B			High vit. B high protein		
Added Fat	Nil	5% U.S.	5% S.	Nil	5% U.S.	5% S.	Nil	5% U.S.	5% S.
Mean Shank Length									
8 weeks	83.2	81.3	80.4	82.2	81.7	80.8	87.6	87.5	89.8
12 weeks	106.3	103.1	106.2	106.3	103.0	103.6	110.2	109.5	110.5
Means for Basal Diets									
at 8 weeks		81.6			81.6			88.3	
at 12 weeks		105.2			104.2			110.1	
Means for Fat Treatments		*No Fat*			*5% U.S.*			*5% S.*	
at 12 weeks		107.7			105.6			106.8	

Least significant difference between means of 3 treatments at 8 weeks (P = 0.05) 1.6
(P = 0.01) 2.2

Least significant difference between means of 3 treatments at 12 weeks (P = 0.05) 1.8
(P = 0.01) 2.4

U.S.—Unstabilized Fat.
S.—Stabilized Fat.

TABLE 9.—*Feed conversion ratio (lb. feed/lb. gain) between 4½ and 8 weeks*

Added Fat	Nil			5% unstabilized			5% stabilized		
Basal Diets	N.	H.B.	H.B.P.	N.	H.B.	H.B.P.	N.	H.B.	H.B.P.
Conversion Ratio	3.24	3.11	3.24	2.92	2.93	2.88	2.94	2.91	2.89
Means for Fat Treatments		3.163			2.910			2.913	
Least significant difference between fat treatments (P=0.05) 0.113									
(P=0.01) 0.188									

N.—Normal.
H.B.—High Vitamin B.
H.B.P.—High Vitamin B plus High Protein.

termined during this period. There was a highly significant advantage in favor of the diets containing fats but no significant variation between the three basal diets. Because of the limitations in the experimental design it was not possible to measure any interaction between basal diets and fat supplements in determining feed conversion, but such effects are evidently small compared with the effect of fat (mean square for fat = 3.25 times mean square for interactions).

Experiment 2.—Because of the small number of degrees of freedom in the analysis of feed conversion, no significant effects were obtained. Table 10 shows the efficiencies of feed conversion between 8 and 12 weeks.

TABLE 10.—*Feed conversion ratio (lb. feed/lb. gain) between 8 and 12 weeks*

Basal diet	0 fat	Low protein	5% fat	Low protein	5% fat	High protein
0.10% Methionine	Nil	+	Nil	+	Nil	+
Conversion Ratio	6.63	5.12	5.91	4.91	4.88	4.73

DISCUSSIONS AND CONCLUSIONS

These experiments confirm the literature in that the effect of tallow is to improve the feed conversion ratio, measured as pounds of feed consumed per pound of grain. Tallow had no effect on the body weight or rate of growth of these birds. Bigbee *et al.* (1957) reported that fat increased growth rate as well as feed efficiency in cool weather, but only feed efficiency in summer. Other reports show no improvement in rate of gain (Siedler *et al.*, 1955), or no consistent increase in growth rate (Curtin and Raper, 1956) due to fat supplementation. The present experiments were carried out during summer. At a level of 5% as fed in these experiments, no harmful effects were noted so that when the economics are sound, tallow should be a useful ingredient of a broiler mash.

Stabilization of the tallow with antioxidant had no significant effect on body weight or feed conversion at any stage during these experiments. It is possible that with lower grade tallows, stabilization will show a response. Further work with lower grade fats is planned at this Station. Such lower grade fats may well prove to be economic.

An unexpected result of the stabilization of tallow was revealed on analysis of variance of shank length at 12 weeks. Unstabilized fat from 0 to 8 weeks, even though it had shown no effect at 8 weeks, showed a slight but significant ($P < 0.05$) decrease in the 12 weeks shank length. The shank length of the birds receiving stabilized fat to 8 weeks was lower but not significantly so, than that of those with no fat. Vitamin E antagonism by unstabilized fat may play a part but no explanation is attempted without further supporting work.

The present experiments have demon-

strated a striking response to high protein in growing diets. Present locally recommended standards of 20% protein for starting diets and 15% protein from 6 weeks onwards, appear below optimum for growing poultry. This will probably apply more particularly in the trend towards high energy diets. Twenty-five percent protein up to 8 weeks produced a large increase in body weight above that of the birds on the recommended 20% protein, which occurred both in presence and absence of fats. This initial increase in body weight did not diminish gain between 8 and 12 weeks. Nineteen percent protein between 8 and 12 weeks again showed significant improvement over the recommended 15% level. Absence of interaction between treatments before, and treatments after 8 weeks, indicates that rapid early growth did not impose any limit to growth after 8 weeks. It is conceivable that such an interaction where rapid early growth limits subsequent growth may be present at a later age.

These results disagree with American reports (Lloyd, Reed and Fritz, 1949) that protein levels above 20% may give faster early growth of chicks with the advantage being largely lost by market age. There is no doubt that in the present experiments, high protein levels in experiment 1 had a lasting effect which had not diminished at 12 weeks even when the protein level was reduced to 15% for the length of experiment 2.

The independent variation between treatments, of shank length and body weight, suggests that some differences in tissue growth and carcass composition occurred between treatments. Shank length may be regarded as a measure of skeletal growth, *i.e.* growth of a body tissue unaffected by fat deposition. Body weight includes fat deposition.

Up to 8 weeks (experiment 1) protein level showed the dominant effect on both shank length and body weight measurements. In the period between 8 and 12 weeks (experiment 2), shank length increase was no longer affected by differences in basal diets (protein and energy) whereas body weight responded to basal diets and methionine supplementation. Shank length increase showed a negative interaction between basal diets and methionine response, the methionine response being positive at low protein and energy levels, and negative at high protein and energy levels. This may be explained by the requirements of folic acid in protein synthesis (Stockstad *et al.*, 1954; Brachet, 1955) and in catabolism of methyl groups (McDonald, 1957). As the added methionine is broken down to cysteine the folic acid requirements for removal of methyl groups increase. If the diet is marginal in folic acid, and previous work at Seven Hills (McDonald, 1957) suggests this, then the lowered intake of folic acid with the high energy diet affects muscular and skeletal growth measurements while body weight need not show this depression because fat deposition can compensate the effect. This explanation would confirm the literature in that lower total feed consumption of high energy rations can make marginal nutrient levels deficient.

SUMMARY

Experiments were carried out to determine the value of tallow and the effect of protein level in all mash broiler diets based on wheat meal.

Added fat did not affect growth rate but it improved feed conversion when measured as lbs. of feed consumed/lb. gain. Stabilization of the fat had no effort on body weight or feed efficiency.

Birds fed protein levels of 25% to 8 weeks and 19% from 8 to 12 weeks showed considerably better growth rate than birds fed protein levels of 20% and 15% respectively. The weight increase shown at 8

weeks was not diminished at 12 weeks even where birds were fed at the lower protein level between 8 and 12 weeks.

That differences in carcass composition were produced between treatments was suggested by independent variation of shank length and body weight.

ACKNOWLEDGEMENTS

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UTILIZATION OF FAT BY YOUNG PIGS: FATTY ACID COMPOSITION OF INGESTA IN DIFFERENT REGIONS OF THE DIGESTIVE TRACT AND APPARENT AND CORRECTED DIGESTIBILITIES OF CORN OIL, LARD AND TALLOW

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ABSTRACT

Young pigs of 13 to 22 days of age, weaned at 7 days, were used to determine the digestibilities of corn oil, lard and tallow; the mean corrected digestibilities were 86, 81 and 56%, respectively. At 22 days of age, the animals were slaughtered and the fatty acid composition of samples from the contents of six different regions of the digestive system were examined by gas-liquid chromatography. The contents of the digestive systems of a conventional and a germ-free pig which had been receiving the same diet were also examined. The low digestibility of stearic acid and the high digestibility of oleic and linoleic acids appeared to be a result of microfloral activity rather than a direct result of the pig's ability to absorb these fatty acids.

RESUME

On a déterminé la digestibilité de l'huile de maïs, du saindoux et du suif sur des porcelets de 13 à 22 jours d'âge, sevrés à 7 jours; les digestibilités vraies moyennes ont été de 86, 81 et 56%, respectivement. A 22 jours les animaux ont été abattus et la composition en acides gras d'échantillons du contenu de six régions différentes du tube digestif a été déterminée par chromatographie gaz-liquide. Les contenus du tube digestif d'un porcelet normal et d'un porcelet axénique ("germ-free") qui avaient reçu le même régime ont été également examinés. La faible digestibilité de l'acide stéarique, ainsi que la haute digestibilité des acides oléique et linoléique, paraît être un artéfact provenant de l'activité de la microflore plutôt que le résultat direct de la capacité du porc à absorber ces acides gras.

INTRODUCTION

There is little experimental evidence of an advantage in feeding high fat diets to early-weaned pigs (10). When high fat diets are fed, the digestibility of the added fat is generally low and feed intake is often reduced, which depresses growth rate. However, sow's milk contains 30 to 40% fat on a dry matter basis (6), and this is well digested (7); therefore, investigations of the factors which influence fat digestibility in the young pig would be useful.

The objectives of the experiment reported here were to measure the digestibility of three fats: corn oil, lard and tallow, and to follow the changes in the fatty acid composition of the ingesta as it passed through the gastro-intestinal tract of the young pig.

MATERIALS AND METHODS

Sixteen Yorkshire × Lacombe piglets from two litters were weaned at 7 days of age and were housed individually in cages which allowed separate collection of urine, feces and waste feed. The temperature of the room was maintained between 25 and 28°C; supplemental heating was provided with infrared heat lamps and lighting was continuous. A male and a female from each litter were assigned to each of the four rations (Table 1). Feed and water were provided *ad libitum* and were replaced with fresh material each day.

A preliminary period of 6 days was allowed for the pigs to learn to eat the experimental diets. The feces voided between the 13th and 15th and between the 20th and 22nd days of age were collected and frozen. These samples

Table 1. Details of diet formulation

Ingredients	Basal %	Corn oil* %	Lard† %	Tallow‡ %
Soybean 70% protein concentrate‖	38	38	38	38
Corn starch§	20	20	20	20
Cerelose§	35	25	25	25
Fat	0	10	10	10
Cellulose (Solka Floc)‖	2	2	2	2
Iodized salt	0.5	0.5	0.5	0.5
Calcium phosphate (28% Ca, 22% P)	2.9	2.9	2.9	2.9
Limestone (37% Ca)	0.27	0.27	0.27	0.27
Chromic oxide	0.25	0.25	0.25	0.25
DL-methionine	0.6	0.6	0.6	0.6
Vitamin-mineral supplement¶	0.48	0.48	0.48	0.48
Total	100.0	100.0	100.0	100.0
Composition of diets (determined)				
Protein (N × 6.25) (%)	25.0	25.6	25.8	25.3
Fat (%)	0.9	10.4	10.7	10.9
Dry matter (%)	91.3	92.2	92.5	92.5
Gross energy (cal/g)	4.0	4.5	4.5	4.5

*St. Lawrence Starch Company, Port Credit, Ontario.
†Canada Packers, Toronto, Ontario
‡Skidmore Enterprises, Cincinnati, Ohio.
‖Canada Starch Company, Toronto, Ontario.
§Brown Company, New York, N.Y.
¶The vitamin-mineral supplement supplied the following levels of nutrients per kilogram of diet: vitamin A, 8800 IU; vitamin D_3, 1650 IU; vitamin B_{12}, 33 μg; riboflavin, 7 mg; calcium pantothenate, 17 mg; choline chloride, 1210 mg; niacin, 20 mg; folic acid, 1 mg; menadione sodium bisulfite, 2 mg; thiamine HCl, 2 mg; pyridoxine, 2 mg; D-biotin, 200 μg; santoquin (Delmar), 63 mg; neomycin, 1.4 mg; manganese, 44 mg; copper, 11 mg; iron, 110 mg; zinc, 110 mg.

were freeze-dried and ground for analysis. Dry matter was determined by heating at 105°C for 24 hours. Chromic oxide was determined by the method of Czarnocki *et al.* (5). Gross energy was determined with an oxygen bomb calorimeter.

At 22 days of age the pigs were killed by CO_2 asphyxiation, and their gastrointestinal tracts were removed immediately. Samples of ingesta were taken from six different regions: the stomach, the first half of the small intestine, the second half of the small intestine, the cecum, the colon and the rectum. Each sample was mixed and frozen. The frozen samples were subdivided for analysis; one part (1 to 5 g of dry matter) was used for lipid extraction and another portion was freeze-dried for dry matter and chromic oxide determinations.

Total fat was extracted with chloroform, methanol and water according to the method of Bligh and Dyer (2). The chloroform extract was made up to 100 ml and an aliquot was taken for fatty acid analysis. The remainder of the chloroform extract was reduced to dryness on a rotary evaporator and the residue was weighed.

Approximately 1 mg of the lipid was taken for fatty acid analysis and was transferred to a 16 × 150-mm screw-capped tube for esterification, and 0.2 mg of behenic acid was added as an internal standard. The solvent was removed under reduced pressure on a rotary evaporator using a nitrogen sweep. Ten drops of benzene and 2 ml of 14% boron trifluoride in methanol were added

and the sealed tube was heated to 70°C for 1½ hours. After cooling to room temperature, 4 ml of water was added and the fatty acid esters were extracted with three successive portions of petroleum ether. This was evaporated in a stream of nitrogen and the residue was transferred to a thin layer plate (silica gel G). Benzene was used as the developing solvent and dichlorofluorescein was used to visualize the fatty acid ester band on the thin layer plate. The band was scraped off and extracted with chloroform, dried in a stream of nitrogen and dissolved in 100 μl of carbon disulfide. A suitable amount (2 μl) was separated on a Barber-Colman series 5000 gas chromatograph, equipped with dual columns and dual hydrogen flame ionization detectors. The stainless steel columns, 150 cm × 2 mm inside diameter, were packed with 15% ethylene glycol succinate on 80–100 mesh Gas-Chrom P and were programmed from 160 to 200°C at 4° per minute.

The fatty acid percent composition of the ingesta in the six regions of the digestive tract was investigated in a germ-free pig (maintained in an isolator) and in a pig maintained under the same conditions as those described for the earlier experiment. Both pigs received a liquid diet (SPF-lac) (Borden Company, 350 Madison Avenue, New York 10017).

RESULTS AND DISCUSSION

The dry matter intake and body weight gain of the lard and tallow groups were significantly below those of the basal and corn oil groups (Table 2). However, in all groups there was approximately a 1:1 ratio of dry matter intake to body weight gain. The reduced feed intake of the lard and tallow diets might have been due to the separation of the fat from the rest of the feed in the stomach, which could have caused a delay in gastric emptying. At slaughter, the stomachs of the animals receiving lard and tallow appeared larger than those of the animals receiving either the basal or the corn oil diets. Large globules of fat had separated and collected in the stomachs of the animals fed lard and tallow; in the case of one pig, 65% of the dry matter in its stomach was fat.

The relatively low digestible energy value of the basal ration was due to its low gross energy content, whereas the low digestible energy value of the tallow diet was due to the low digestibility of the dry matter.

Table 2. Dry matter intake, body weight gain, dry matter digestibility, and digestible energy values of the basal diet and the diets containing corn oil, lard and tallow*

Diet	Dry matter† intake (kg)	Gain† (kg)	Dry matter digestibility 2 weeks (%)‡	Dry matter digestibility 3 weeks (%)§	Digestible energy (cal/g DM) 2 weeks‡	Digestible energy (cal/g DM) 3 weeks§
Basal	2.0	2.0	82	81	3.6	3.6
Corn oil	1.8	1.9	82	80	4.0	3.9
Lard	1.4	1.4	80	80	3.9	3.8
Tallow	1.5	1.6	69	73	3.0	3.3

*Mean of four pigs.
†Nine-day period, 13 to 22 days of age.
‡13 to 15 days of age.
§20 to 22 days of age.

Table 3. Utilization of dietary fat: total fat voided from 100 grams of dry feed and apparent and corrected fat digestibilities at 2 and 3 weeks of age*

Fat added	Amount in 100 g of dry feed (g)	2 weeks†			3 weeks†		
		Fat in feces (g)	Digestibility		Fat in feces (g)	Digestibility	
			Appar. (%)	Corr. (%)		Appar. (%)	Corr. (%)
None	0	1.2	—	—	1.4	—	—
Corn oil	10.3	2.3	80	89	3.2	72	83
Lard	10.6	2.9	75	84	3.7	68	78
Tallow	10.8	6.1	48	55	6.2	47	56

*Mean of four pigs.
†See Table 2.

The amounts of fat voided from 100 g of dry feed consumed was determined for the collections taken when the pigs were 2 and 3 weeks of age (Table 3). The mean fat output of the pigs receiving the basal diet was subtracted from the total fat output of each pig receiving the other three diets to indicate the amount of fecal fat derived directly from the dietary fat. It was assumed that the endogenous excretion of fat was the same for all the pigs. This resulted in corrected digestibility values which were approximately 10% higher than the apparent digestibility values. These values are much lower than those reported by other workers; Sewell and Miller (11) found corrected digestibilities of 94% (corn oil), 86% (lard) and 86% (tallow); Cunningham and Brisson (4) found apparent digestibilities of lard ranging between 84 and 91%. However, Eusebio *et al.* (7) used pigs weaned at 17 days and made digestibility determinations at 25 days of age. They found digestibilities of 54% (lard) and 57% (tallow), this value for lard being much lower than the one found in the present study, while the value for tallow was higher. These large discrepancies between the digestibility values reported by different workers indicate that there are factors affecting digestibility determinations in young pigs which have not been controlled. Thus, more of the factors that cause variability in fat digestibility determinations must be eliminated, or controlled, before reliable fat digestibility values of various fats for the young pig can be tabulated.

When the fatty acid composition of the ingesta was investigated throughout the digestive system (Fig. 1), great changes were noticed. There was some increase in the percentage of stearic acid down to the end of the small intestine, but from there the increase in its level was quite dramatic. Conversely, the percentages of oleic and linoleic acids decreased most markedly in the lower digestive system. These changes in concentrations of the fatty acids with 18 carbon atoms cannot be attributed to preferential absorption, since digestion and absorption of fatty acids occur mainly in the duodenum and jejunum of the small intestine and are considered complete by the time the ingested food has passed through the ileum (3). Therefore, the change is attributed to hydrogenation of the unsaturated fatty acids by the microflora (9). However, in previously reported studies the fecal fat has been considered representative of the end product of digestion, and this failure to recognize

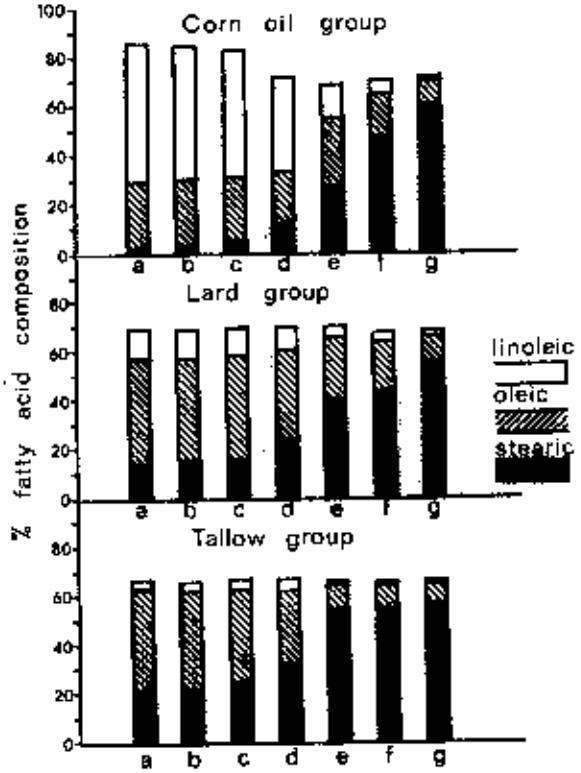

FIG. 1. The levels of stearic, oleic and linoleic acids in the feed (*a*) and ingesta of pigs receiving corn oil, lard and tallow. The six regions of the digestive tract are: stomach (*b*), first half of small intestine (*c*), second half of small intestine (*d*), cecum (*e*), colon (*f*) and rectum (*g*).

the effects of microflora could account for some of the discrepancies between the earlier reports (1, 11) of fatty acid digestibility.

In the present study, the analyses of feed and feces gave very low values for the digestibility of stearic acid and very high values for the digestibilities of oleic, linoleic and linolenic acids (Table 4). It is likely that the wide range in digestibility values obtained for individual fatty acids is not so much a result of the young pig's ability to digest and absorb fatty acids as it is a reflection of the microfloral activity in the lower digestive system.

The chromic oxide indicator tended to separate from the fat in the digestive system, making attempts to determine the absorption of individual fatty acids in the different regions of the digestive system meaningless. This suggests that a fat-soluble indicator would be necessary for quantitative studies of absorption of fat and fatty acids at points within the digestive system.

More evidence of the dramatic effect of the microflora of the lower gut on fatty acid digestibility determinations was obtained from the comparison of the fatty acid compositions of the fat in the digestive systems of the germ-free pig and of the conventional pig, which were both receiving the same diet

Table 4. Fatty acid balance: intake and output (mg/day) from 100 g of dry feed and corrected digestibilities (%)*

Diet:	Basal		Corn oil			Lard			Tallow		
Fatty acid	Input	Output	Input	Output	Corr. dig.	Input	Output	Corr. dig.	Input	Output	Corr. dig.
14:0	1	3	28	3	100	130	10	95	270	6	99
15:0	tr†	16	—	19	—	—	13	—	70	9	110
16:0	58	60	1240	210	88	2760	490	84	2540	370	88
16:1	tr	8	tr	3	400	270	5	101	280	6	101
17:0	tr	12	—	6	—	—	4	—	110	22	91
18:0	25	90	220	690	−170	1570	840	52	2210	810	67
18:1	102	20	2780	120	96	4620	230	95	4130	120	98
18:2	202	4	6010	14	100	1320	8	100	330	2	101
20:0	3	5	106	12	93	5	14	−80	10	18	−30
18:3	30	1	180	tr	100	160	4	98	30	1	100
Unidentified	tr	40	tr	80	—	40	23	140	45	50	78

*Mean of 4 pigs between 20 and 22 days of age.
†Trace, < 0.5 mg/day.

(Fig. 2). The dramatic increase of stearic acid and decrease of unsaturated fatty acids with 18 carbon atoms occurred in the conventional pig, but not in the germ-free pig, indicating that in the conventional pig the microflora is responsible for the pattern of fatty acids that are found in the feces.

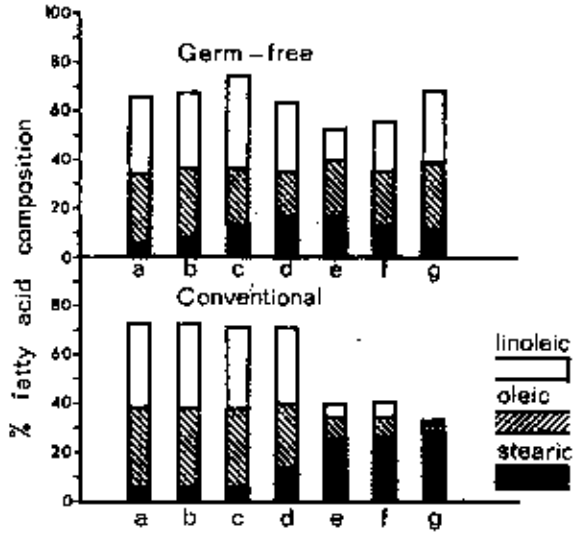

FIG. 2. The levels of stearic, oleic and linoleic acids in the feed (*a*) and ingesta of a germ-free and a conventional pig. The six regions of the digestive tract are: stomach (*b*), first half of small intestine (*c*), second half of small intestine (*d*), cecum (*e*), colon (*f*) and rectum (*g*).

The large discrepancies in values for fat digestibility by the young pig indicate that there are many unknown or uncontrolled factors that affect digestibility determinations. Before meaningful digestibility values can be tabulated, more must be known about these factors so that more control can be used in these determinations. One of the factors that should be given more consideration is the effect the microflora of the lower digestive system has on the fatty acid composition of ingesta once it has passed the regions of fat absorption and before samples are collected for digestibility determinations. This study has shown the marked effect of the microflora on the saturation of fatty acids with 18 carbon atoms, and has suggested that samples taken from the end of the small intestine rather than from the feces would give a truer indication of the pig's ability to digest fat.

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The Chronic Toxicity of Octadecylamine

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The purpose of this investigation was to determine the chronic toxicity of commercial octadecylamine (containing 20% hexadecylamine). This material is of importance as an anticorrosive agent in live steam, which may be used to cook food. It has been estimated that the rate of addition of this chemical to steam is 2.5 ppm, and that the maximum concentration of octadecylamine to be expected in food may range from 0.25 to 0.50 ppm.

The chronic toxicity studies consisted of a two-year rat-feeding experiment and a one-year dog-feeding experiment. In addition to the chronic studies, a short-term experiment comparing the subacute toxicity of octadecylamine with that of stearic acid was conducted, using rats.

Two-Year Rat-Feeding Experiment

Experimental.—One hundred twenty healthy, weanling, Sprague-Dawley rats, housed in individual cages, were used in this experiment. They were divided into five groups of 24 each (12 males and 12 females) with randomization of litter mates among the groups.

The compound dissolved in corn oil was added to ground Purina Laboratory Chow supplemented with 1% cod-liver oil. One group of 24 rats was fed the control diet, the second group received 20 ppm, the third 100 ppm, the fourth 200 ppm, and the fifth 500 ppm octadecylamine. A record was kept of the food consumed by each animal. Body weights were determined weekly. Hematological studies were conducted on animals picked at random from each of the groups. All animals surviving at the end of two years were killed. Pathological examinations were made on the tissues of all animals killed at termination of the experiment and from a few rats killed in extremis. Tissues taken for micropathological examination included brain, pituitary, adrenals, skeletal muscle, bone marrow, heart, lungs, liver, spleen, kidneys, gastroenteric tract (three levels), pancreas, lymph nodes, and ovary or testis.

TABLE 1.—*Food Consumption, Weight Gain, Mortality, and Survival of Rats Fed Octadecylamine for Two Years*

Dietary Concentration Ppm	Sex	Diet Consumption Gm/Day	Mean Weight Gain Gm.	Mortality, per Cent	Mean Survival, Days
0	M	18.9	248.0	67	651.1
	F	14.5	209.4	83	472.4
20	M	17.6	319.6	75	503.6
	F	15.0	198.6	75	540.4
100	M	19.3	291.7	75	608.5
	F	14.5	204.9	88	510.3
200	M	17.8	324.8	75	525.6
	F	12.0	210.9	83	476.5
500	M	18.1	355.5	67	549.3
	F	14.1	204.4	83	504.5

Results.—There was no significant difference observed between the mean daily consumption of experimental and control diets (Table 1). The mean weight gains were also essentially the same for all groups. There was no significant difference between the animals fed octadecylamine and the controls in either the percentage mortality or the mean survival time. In both control and experimental rats there was a higher than expected rate of mortality due to acute respiratory infections. Numerous attempts were made to culture the organisms responsible for these deaths. However, only normal bacterial inhabitants of a rat's respiratory

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#4 TABLE 2.—*The Effects on the Peripheral Blood of Feeding Octadecylamine to Rats for Two Years*
(Average values and standard deviations of counts taken on groups of five or six rats after 96 weeks of feeding)

Dietary Concentration Ppm	Hemoglobin Gm/100 Ml. Mean±S. D.	Red Blood Cells Millions/Cu. Mm. Mean±S. D.	White Blood Cells Thousands/Cu. Mm. Mean±S. D.	Neutrophils Thousands/Cu. Mm. Mean±S. D.	Lymphocytes Thousands/Cu. Mm. Mean±S. D.
0	11.6±1.1	5.2±1.0	21.8±2.2	6.0±2.9	13.7±3.7
20	12.8±0.2	6.5±1.7	23.4±8.3	5.7±5.6	14.6±4.3
100	13.2±1.9	5.1±0.7	22.9±5.9	7.6±1.8	14.9±3.3
200	12.3±1.5	4.9±0.7	17.0±4.8	6.2±2.0	12.7±5.2
500	14.5±3.4	5.6±1.3	19.5±3.2	5.3±2.4	13.7±3.3

tract were found. The impression was gained that these deaths were the result of a nonspecific endemic pulmonary infection occasionally observed in rat colonies.

The blood studies were conducted on all groups of rats after 4, 8, 13, 16, 20, 25, 28, 43, 55, 66, 86, and 96 weeks of feeding. The only significant change that was noted was a slight upward trend in the total white and total neutrophil count. This may have been associated with the prevalence of respiratory disease in the colony. The final (96-week) values shown in Table 2 reveal no significant differences in hemoglobin concentration, red count, white count, absolute neutrophil count, and absolute lymphocyte count between any of the groups fed octadecylamine and the controls.

Inasmuch as a preliminary study (to be reported below) with doses of 3000 ppm of octadecylamine had shown a constant and striking change in the gastroenteric tract and mesenteric lymph nodes, particular attention was focused on these organs as the sites of expected micropathologic change. However, no change, either quantitatively or even qualitatively similar, was observed.

There were no significant pathologic differences between the control group and the groups of rats fed 20, 100, 200, and 500 ppm octadecylamine. Lesions encountered in all groups included pyelonephritis, hyaline casts in the renal tubules, occasional renal abscesses, and bronchitis or pneumonia. One animal in the control group showed a mild endocarditis and valvulitis; one animal in the 100 ppm octadecylamine group developed a brain abscess, and one animal receiving 500 ppm showed histocytic hyperplasia of a mesenteric lymph node. The changes observed were not considered significant or related to the ingestion of octadecylamine.

One-Year Dog-Feeding Experiment

Twelve young mongrel dogs were selected for the one-year dog feeding experiment. They were housed in individual cages and fed once daily. The diet consisted of a mixture of Purina Kibbled Meal and canned horse meat. In addition, they had access at all times to Hunt Club Bones (a dog biscuit). The twelve dogs were divided into four groups of three animals each. One group (two males and one female) served as control; a second group (one male and two females) received octadecylamine at a dose level of 0.6 mg/kg/day; the third group (two males and one female) 3.0 mg/kg/day, and the fourth group (one male and two females) 15.0 mg/kg/day. Throughout the experiment, octadecylamine in corn-oil solution was administered by capsule once daily except Saturdays and Sundays. The dogs were weighed once a month and the doses recalculated on the basis of the new weights. Hematological examinations consisting of total red blood cell count, hemoglobin concentration, and total and differential white cell counts were performed on the 12 dogs at the start of the experiment and 1, 2, 3, 6, 8, 11, and 12 months thereafter. All surviving animals were killed at the end of the year for gross and microscopic examination.

Results.—With one exception, all dogs survived the experiment in obvious good

TABLE 3.—*Weight Changes of Dogs Fed Octadecylamine for One Year*

Identification No.	Sex	Daily Dose of Octadecylamine Mg/Kg.	Initial Weight of Dog Kg.	Final Weight of Dog Kg.	Change of Weight Kg.
D-89	M	0	10.2	12.8	+2.6
D-91	F	0	4.8	9.6	+4.8
D-97	M	0	7.6	12.2	+4.6
				Average gain	4.0
D-96	F	0.6	10.4	11.7	+0.7
D-95	M	0.6	9.7	11.6	+1.8
D-98	F	0.6	5.7	10.3	+4.6
				Average gain	2.4
D-93	F	3.0	8.1	13.8	+5.7
D-92	M	3.0	6.0	7.8	+1.4
D-99	M*	3.0	7.5	12.6	+5.1
				Average gain	4.2
D-90*	M	15.0	6.4	6.0	−0.4
D-94	F	15.0	7.0	7.5	+0.5
D-100	F	15.0	8.5	9.7	+1.2
				Average gain	0.4

* Died after 22 weeks.

health. One dog, at the 15 mg/kg. level, died after 22 weeks. This animal suffered from anorexia during the last three weeks of life, and exhibited bloody diarrhea during the three days prior to death. The animal did not show a severe loss in weight, but the impression was gained that it was suffering from gastroenteric irritation.

The mean weight gain of the dogs receiving 15 mg/kg. was less than that gained by the control dogs or those fed lower levels of octadecylamine. The weight gain of the dogs on both of the lower levels was essentially the same as that of the control dogs (Table 3).

Blood studies were performed at the beginning of the experiment and after 1, 2, 3, 6, 8, 11, and 12 months of drug administration. A slight gradual increase in the total number of red blood cells and concentration of hemoglobin was observed as the experiment progressed. These changes were noted in all control and experimental dogs, and were expected since the dogs used for the experiment were young and attained maturity as the experiment progressed. The initial and final values are shown in Table 4. There are no significant differences between the animals receiving octadecylamine and the controls.

Gross pathological organ changes were not observed. The organs examined microscopically included the heart, lungs, liver, spleen, kidneys, gastroenteric tract (three

TABLE 4.—*Effect of Feeding Octadecylamine to Dogs for One Year*
(Only initial and final counts are given; complete blood counts were taken after 1, 2, 3, 6, 8, and 11 months of feeding)

Identification No.	Hemoglobin Gm./100 Ml.		Red Blood Cells Millions/Cu. Mm.		White Blood Cells Thousands/Cu. Mm.		Neutrophils Thousands/Cu. Mm.		Lymphocytes Thousands/Cu. Mm.	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control Dogs										
D-89	10.4	11.8	5.2	5.6	10.9	13.3	6.5	9.7	3.9	3.9
D-91	11.4	12.2	5.4	6.8	12.2	9.4	9.4	4.1	2.8	5.0
D-97	11.0	14.4	6.3	7.8	10.4	8.4	3.9	4.0	5.6	2.2
Daily Dose: 0.6 Mg/Kg.										
D-96	12.4	15.4	5.4	7.5	5.8	9.9	4.1	6.3	1.2	3.3
D-98	12.3	12.2	5.9	6.4	8.4	17.6	5.9	9.4	0.4	7.4
D-78	13.0	14.6	5.2	7.0	10.9	11.9	4.8	7.0	5.4	3.8
Daily Dose: 3.0 Mg/Kg.										
D-92	11.0	13.6	6.0	7.2	8.9	12.8	7.6	6.7	0.7	5.9
D-93	10.2	14.6	5.8	7.6	13.6	9.0	9.9	5.5	2.6	4.1
D-99	11.0	14.8	6.5	8.2	7.5	10.3	3.9	6.2	3.2	3.5
Daily Dose: 15.0 Mg/Kg.										
D-90	11.4	16.6*	6.1	5.8*	8.6	8.2*	4.0	5.8*	2.2	1.0*
D-94	11.8	16.0	6.2	8.2	6.4	13.6	5.0	9.8	1.1	3.3
D-100	11.4	17.0	6.0	8.5	7.4	7.8	3.7	5.0	3.0	1.7

* These final counts after 6 months of feeding (dog died subsequently)

levels), pancreas, lymph nodes, bone marrow, brain, pituitary, adrenals, and ovary or testis. No definite lesion of the intestinal tract was identified. However, in the two surviving animals of the group that received 15.0 mg/kg. of octadecylamine, the tips of the villi of the mucosa of the small intestine were pale-staining. This appeared in the superficial substantia propria. No foam cells or deposits of lipid material were identified. The intestines of these dogs had a different appearance from the intestines of the other dogs and were slightly similar to those of rats fed 3000 ppm octadecylamine, suggesting the possibility of some absorption of a nonstaining material into the mucosa of the small intestine. Also, in both of these animals the sinuses of the mesenteric lymph nodes were filled with pale, "foamy" histiocytes.

Comparison of Subacute Toxicity of Octadecylamine with That of Stearic Acid

One group of 10 Sprague-Dawley rats (5 males and 5 females), housed in individual cages, was fed 3000 ppm of octadecylamine in the basic diet. A similar group received the basic diet containing 3000 ppm of stearic acid (a straight-chain fatty acid differing from octadecylamine only in that it contains a carboxyl group in place of the terminal primary amino group). These experimental diets were fed for a total of 209 days. A record was kept of the food consumed by each animal. Individual body weights were determined weekly throughout the experiment. All animals surviving at the end of 209 days were killed. Gross and microscopic pathological examinations were made on tissues taken from all animals killed at termination of the experiment and from certain animals killed in extremis while the experiment was in progress. Tissues taken for micropathological examination included brain, viscera, gonads, skeletal muscle, bone marrow, heart, lungs, liver, spleen, kidneys, gastroenteric tract (three levels), pancreas, and lymph nodes.

TABLE 5.—*Effect of Feeding to Rats 3000 Ppm of Octadecylamine and 3000 Ppm of Stearic Acid for a Period of 209 Days*

Compound	Sex	Mortality Ratio	Average Survival Time, Days	Average Weight Change, Gm.	Average Diet Consumption per Day, Gm.
Octadecylamine	Males	4/5	87	−31	9.8
	Females	1/5	119	+30	11.5
Stearic acid	Males	5/5	107	+12	15.0
	Females	3/5	127	+58	13.5

Results.—Both groups exhibited anorexia, but the rats that received octadecylamine had a significantly lower mean daily intake of food than those receiving stearic acid in their diet (Table 5). The males on octadecylamine showed a mean weight loss of 31 gm. while those fed stearic acid gained 30 gm. Although the females on octadecylamine showed a mean gain of 30 gm., it was significantly less than the gain of the females on stearic acid, which was 58 gm. The mean period of survival of both males and females receiving octadecylamine was shorter than that for the rats receiving stearic acid. On the other hand, the mortality ratio of the rats on stearic acid was slightly greater for both males and females. It is interesting to note that all of the rats on octadecylamine lost weight initially. However, those which survived began to gain midway during the course of the experiment. This was not true of the rats on stearic acid: throughout the experiment these animals showed a gradual but somewhat erratic tendency to gain.

Tissues from rats fed 3000 ppm stearic acid showed severe pulmonary infection consisting of tracheobronchitis, lobular pneumonia, lipoid histiocyte response, and abscess formation. No retention or imbibition of this ingested material was noted in sections of the gastroenteric tract. Abundant iron-containing pigment was found in the spleens; the other organs examined microsopically, including the heart, liver, kidneys, pancreas, and adrenals, did not show significant abnormalities.

Tissues from rats fed 3000 octadecylamine showed varying degrees of pulmonary infection. Sections from the gastroenteric tract revealed a diffuse aggregation of foamy histiocytes within the mucosa of the small intestine in each animal. These were not stained by Sudan IV. The mesenteric lymph nodes were grossly enlarged and matted. Microscopic examination of them revealed a marked granulomatous reaction with abundant histiocytes, nodule or tubercle formation, necrosis, and fibrosis. These lymph node sections stained weakly positive with Sudan IV. Sections of the livers of three of five animals showed nodular aggregates of histiocytes, with slight necrosis. This feature was most pronounced in the two animals that survived for the shortest period of time (118 and 133 days). In the third animal (137 days) only one such nodule was seen. Although the mesenteric nodes of all five animals showed a granulomatous response, this likewise appeared more intense in rats fed octadecylamine for 118 and 133 days. The other organs examined—heart, spleen, pancreas, kidneys, and adrenals—were normal.

Summary and Conclusions

No toxic effects were observed when, for a period of two years, octadecylamine was fed to rats in the diet at levels up to and including 500 ppm. The weight changes, blood counts, percentage and distribution of mortality, and food consumption of the rats fed octadecylamine were similar to those noted in the controls. Octadecylamine at this level produced no micropathological changes in rats.

No toxic effects were observed in dogs given octadecylamine at levels of 0.6 and 3.0 mg/kg. per day on each of five days of a week for a period of 12 months. One of the three dogs receiving 15 mg/kg/day died after 22 weeks. The other two dogs gained less weight than controls or the dogs receiving the lower levels of octadecylamine. Two of the three animals fed 15.0 mg/kg. (including the one which died) showed slight changes of an equivocal nature in the mucosa of the small intestine. Otherwise, microscopic examination of the tissues showed no pathology. While the death of the one dog might be fortuitous, the bulk of the evidence indicates that octadecylamine has some toxic effects (primarily on the gastroenteric tract when fed at a level of 15.0/kg/day). Thus it appears that the maximum nontoxic levels of octadecylamine are 500 ppm for the rat, and 3.0 mg/kg/day (equivalent to approximately 100 ppm in the diet) for the dog.

The preliminary experiment on the comparative subacute toxicity of octadecylamine and stearic acid (a compound with the same carbon skeleton but having a carboxyl group substituted for the terminal primary amine group) demonstrated that at a level of 3000 ppm in the diet the two compounds elicited similar toxic reactions. Both compounds produced anorexia and increased rate of mortality. Rats fed stearic acid showed no significant pathological lesions, but those fed octadecylamine showed an accumulation of histiocytes with pale or foamy cytoplasm in the mucosa of the small intestine and mesenteric lymph nodes. Focal granulomas were seen in the livers of some of these latter animals.

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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

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	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	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.

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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Factors Affecting the Absorbability of Certain Dietary Fats in the Chick[1,2]

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The presence of fat in the diet of the chicken has a beneficial influence on the efficiency of feed utilization (Sunde, '56, and others) and frequently on growth rate (Waibel, '55, and others). The chick has the ability to utilize a high proportion of its energy requirement in the form of fat (Rand et al., '58; Donaldson et al., '57).

Certain fats, however, are poorly absorbed by animals (Deuel et al., '40; Hoagland and Snider, '40). Thus, the low metabolizable energy values of animal tallow for chicks, as compared with certain vegetable oils, as found by Renner and Hill ('58), is of major importance in terms of energy conservation.

Factors which have been shown to modify the absorbability of certain fats include the age of the animal (Duckworth et al., '50; Renner and Hill, '58) and the presence of dietary mineral ions (Givens and Mendel, '17; Cheng et al., '49). The work to be reported herein compared, in the chick, the absorbabilities of certain fats and studied factors influencing the absorbability of a poorly utilized fat, namely, beef tallow. Factors studied were the age of bird, dietary addition of ox bile, restriction of feed intake and dietary calcium level.

EXPERIMENTAL

Male crossbred chicks from Dominant White ♂♂ × White Plymouth Rock ♀♀ were used in experiments 1 (10 per group) and two (7 per group), and from Vantress ♂♂ × Nichols 108 ♀♀ were used in experiments 3 (5 replicates of 5 chicks each) and 4 (4 replicates of 5 chicks each). Chicks were wing banded and placed randomly in standard electric starting batteries with raised wire-mesh floors until 4 weeks old. If the experiment proceeded longer than 4 weeks, the chicks were transferred to growing batteries.

Fat absorption trials were conducted for a three-day period during which the feed eaten and feces (total excrement including urine) excreted were quantatively measured. Wet feces were ground with the aid of an inert water absorbent.[3] Care was taken to prevent moisture loss from the feces between the time of collection and the time of analysis. The chemical analysis for fat in the feces was conducted according to the procedure described by Van de Kamer et al. ('49). The results were calculated as grams of fatty acids per 100 gm of feces. This value represents *both neutral fat and fatty acids excreted* but will be reported as fatty acids excreted, only because this is the value given by the chemical analysis. The same chemical procedure was used to determine the fat content of the feed. Apparent absorption coefficients were calculated according to the formula:

$$\frac{\text{Fat consumed} - \text{fat excreted}}{\text{Fat consumed}} \times 100.$$

Body weight and feed efficiency data were collected weekly. Feed efficiency was calculated by dividing the feed consumed by total weight gain.

Analyses of variance were used in experiments 3 and 4 to evaluate the differences, according to Snedecor ('56). If significant differences occurred, the Student-Newman-Keuls Multiple Range Test was used to locate them (Federer, '55).

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[2] From a thesis submitted by the senior author to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements of the M.S. degree, August, 1959.

[3] Micro-Cel E, Johns-Mansville, New York.

TABLE 1
Composition of diets

Ingredient	Basal	10% fat	20% fat
	%	%	%
Corn, ground yellow	63.25	45.25	26.25
Soybean oil meal, dehulled	24.75	32.75	41.75
Fat[1]	0	10.0	20.0
Constant ingredients[2]	12.0	12.0	12.0
Calculated analysis:			
Protein, %	20.76	23.31	26.29
Productive energy,[3] Cal./pound	926	1046	1161
Calorie (P.E.), % protein ratio	44.6	44.9	44.1
Calcium, %	1.24	1.27	1.29

[1] Beef tallow, hog grease, non-break safflower oil and refined corn oil were used. The value of 2760 Productive Energy Calories per pound was used for all fat sources.

[2] Constant ingredients. In per cent: corn distillers' dried solubles, 2; fish meal (60% protein), 3; alfalfa meal (dehydrated, 17% protein), 1; dried whey, 2; dicalcium phosphate, 2; calcium carbonate, 1.25; iodized salt, 0.5; vitamin A (10,000 I.U./gm), 0.03; vitamin D^3, (3000 I.U./gm), 0.02; d-α-tocopheryl acetate (44 I.U./gm), 0.01; choline chloride (70% soln.), 0.09; and DL-methionine, 0.05. In milligrams per kilogram of diet: chlortetracycline·HCl, 25; biotin, 0.2; menadione sodium bisulfite—63% MSB(USP), 2; pyridoxine·HCl, 2; folic acid, 2; thiamine·HCl, 2; riboflavin, 4; calcium pantothenate, 10; niacin, 20; and vitamin B_{12} (0.1% triturate), 40. In experiment 1, 0.04% manganese sulfate (feeding grade) was used. In experiment 2, 3 and 4, 0.10% of a trace mineral mixture (Delamix-Limestone Products Corporation of America, Newton, New Jersey) was used and provided the following in milligrams/kilogram of diet: manganese, 60; iodine, 1.2; iron, 20; copper, 2; zinc, 0.06; and cobalt, 0.2.

[3] Fraps, '46.

The diets used are presented in table 1.[4] The ratio of productive energy to protein was held constant, thus the only variables in the diet were corn, soybean oil meal and fat.

RESULTS

Experiment 1 was conducted to determine the apparent absorbabilities of some natural fats and oils (table 2). Essentially no differences in growth were evident among groups receiving different types and levels of fats or oils and the group receiving the basal diet. As expected, there was an improvement in feed efficiency, over the basal group regardless of the type or level of fat or oil fed.

[4] The authors wish to thank the following firms for their generous supply of experimental materials: Armour and Company, Chicago; Van Hoven Company, Inc., St. Paul, Minnesota, and Pacific Vegetable Oil Corporation, San Francisco.

TABLE 2
Utilization of dietary fats by chickens

Diets	Weights		Feed/gain		Apparent absorbability		Fatty acids excreted /100 gm fed	
	4 weeks	8 weeks	4 weeks	8 weeks	1–2 weeks	7–8 weeks	1–2 weeks	7–8 weeks
	gm	gm			%	%	gm	gm
Basal, no added fat	441	1360	1.91	2.21	71.8	76.9	0.84	0.62
10% Safflower oil	441	1361	1.60	1.92	92.7	93.1	0.78	0.73
10% Corn oil	447	1391	1.81	2.05	85.0	90.7	1.72	1.07
10% Hog grease	425	1327	1.65	1.90	84.8	89.8	1.82	1.22
10% Beef tallow	437	1344	1.76	2.05	59.1	74.3	4.92	3.10
20% Safflower oil	429	1365	1.76	1.93	89.7	91.9	2.04	1.60
20% Corn oil	432	1426	1.63	1.98	90.2	92.4	2.01	1.98
20% Hog grease	469	1325	1.49	1.77	85.4	93.0	2.98	1.44
20% Beef tallow	452	1386	1.77	1.96	46.6	78.6	11.20	4.49
20% Beef tallow + 0.5% ox bile	424	1391	1.73	1.81	68.9	88.9	6.38	2.29

Very little fat was excreted in the feces when diets contained 10 or 20% of corn oil, safflower oil or hog grease. However, when 20% of beef tallow was fed, a large quantity of fat was excreted, especially at the early age. There was an increase in beef tallow absorbability from two to 8 weeks. The group fed 20% of tallow plus 0.5% of ox bile, absorbed the tallow more completely than the group receiving only 20% of tallow.

Experiment 2 was conducted to determine whether the increase in absorbability of beef tallow with age was due to age-mediated changes or to an adaptability of the gut (in the form of increased enzyme concentration, altered microflora, increased bile flow, and others) to the fat. Growth and feed-efficiency data are omitted since differences conformed to expectation.

Of 10 groups placed on experiment, 5 were fed the basal diet, one the 20% beef tallow diet and two the 20% safflower oil diet. Then, at two, 4, 6 and 8 weeks of age, certain groups were switched from the basal or safflower oil diets to the beef tallow diet, as shown in figure 1. Since variability was small among results of identical treatments, the data are combined into one line for the benefit of simplicity in presentation. The two other treatments in this experiment (not shown) were a 10% beef tallow-10% hog grease combination and a 20% beef tallow group switched to 10% beef tallow at 6 weeks of age.

The birds which received the basal and safflower oil diets excreted very little fat when compared with groups fed the beef tallow diets. The amount of fat excreted by the groups fed beef tallow decreased with increasing age. When 20% beef tallow diets were substituted for the basal diets, at the various ages, the amount of fat excreted was approximately the same as that excreted by the respective birds fed the 20% tallow diet continuously.

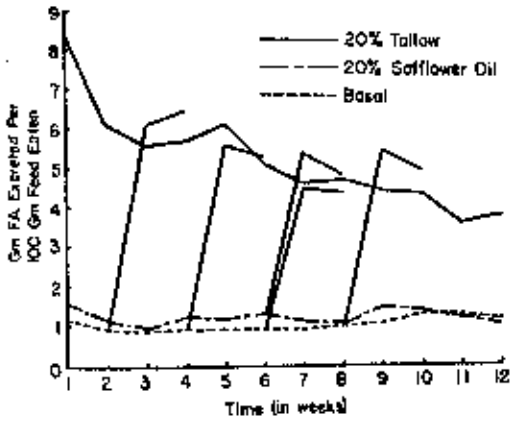

Fig. 1 Effect of age on fat absorbability. (See results section for details.)

When the 20% beef tallow diet was changed to a 10% beef tallow diet at 6 weeks, the birds exhibited no increase in apparent fat absorbability. The group which received 10% each of beef tallow and hog grease gave absorbability results intermediate to the 20% beef tallow and 20% safflower oil groups.

Experiment 3 was designed to study the influence of graded levels of ox bile on the fat absorbabilities of diets containing 20% of beef tallow (table 3). The 4 and 8% ox bile levels caused a significant growth depression throughout the experiment. Feed efficiencies paralleled growth, namely, when 4 and 8% ox bile levels were fed, feed was least efficiently utilized.

Greatly enlarged gallbladders were found in birds fed 0.5% of ox bile in experiment 1. Thus, to verify this observation, the bile in the gallbladders was removed and measured with a calibrated syringe at the end of the 4-week period in this experiment. It was assumed that the amount of bile present was indicative of the gallbladder size. Visual observations indicated that the gallbladders of birds that received high levels of ox bile were greatly enlarged when compared with the other organs, such as the liver. The results in table 3 show that gallbladders of birds fed the 8% ox bile diet were relatively larger than those of birds fed any other diet and that ox bile levels over 1% resulted in a significant increase in bile storage.

Although the apparent absorbability of the 20% beef tallow diet was higher than had been found previously, a significantly higher absorbability of fat was found when ox bile levels of 0.5% and higher were fed. No significant difference in the absorption coefficients existed between 8, 4, 0.5 and 1% ox bile levels. This suggests that 0.5% of ox bile is virtually as effective in increasing fat absorption as is the high 8% level.

TABLE 3

Effect of dietary ox bile on growth, gallbladder size and fat absorption of chicks fed 20% of beef tallow

Four-week weights:								
Ox bile level, %	0	0.1	1	2	0.5	0.05	4	8
Means,[1] gm	611	597	577	575	567	556	409	243
Ratio of CC. bile in gallbladder to body weight (in gm) × 100:								
Ox bile level, %	0	0.1	0.05	0.5	1	2	4	8
Means[1] of ratio	0.11	0.14	0.18	0.18	0.20	0.20	0.25	0.38
Apparent absorption coefficients:								
Ox bile level, %	0	0.1	0.05	2	1	0.5	4	8
Means,[1] 21–23 days, %	83	84	85	86	87	88	89	90

[1] Any two or more means not underscored by the same line are significantly different at the 0.05 level of probability. See text for methods.

TABLE 4

Effect of restricted feed intake and dietary calcium levels on growth, feed efficiency and fat absorption of chicks fed 20% of beef tallow

Four-week weights:					
Treatment	Low Ca	Control	High Ca	80% AL[1]	60% AL[1]
Means,[2] gm	539	524	480	431	336
Four-week feed efficiency:					
Treatment	High Ca	Control	80% AL[1]	60% AL[1]	Low Ca
Mean,[2] feed/gain	1.92	1.74	1.71	1.71	1.63
Apparent fat absorption coefficients:					
Treatment	Low Ca	Control	80% AL[1]	60% AL[1]	High Ca
Mean,[2] 11–14 days, %	68	71	71	71	56
Means,[2] 25–28 days, %	91	77	76	75	71

[1] Ad libitum.
[2] Any two or more means not underscored by the same line are significantly different at the 0.05 level of probability. See text for methods.

In experiment 4, the effects of restricted feed intake and dietary calcium level on growth, feed efficiency and fat absorbability by chicks were measured (table 4). The treatments used were (1) control diet, 20% of beef tallow, normal calcium (1.24%), ad libitum; (2) control diet fed at 80% ad libitum after 7 days of full feeding, the intake being calculated from the preceding day's consumption by the full-fed controls; (3) control diet fed at 60% ad libitum, carried out the same as (2); (4) control diet in which the calcium level was raised to 3% during the entire experiment by adding 45 gm of calcium carbonate per kg of diet; and (5) control diet, in which the calcium level was lowered to 0.33% during the digestion trials

(4 days, i.e., one day preceding and 3 days during trial) by removing calcium carbonate and dicalcium phosphate. The total phosphorus level was lowered to 0.49% during the periods when this diet was fed.

Birds fed restricted diets had lower body weights than the controls, in accordance with the degree of restriction. However, restricted feed intake caused no change in feed efficiency or the ability of the birds to absorb beef tallow when compared with the controls. A high 3% dietary calcium level depressed growth and feed efficiency somewhat, and also lowered the absorbability of beef tallow at two and 4 weeks of age. Growth or efficiency of feed utilization was not altered, over that of the control, by lowering the calcium level to 0.33% and the phosphorus level to 0.49% during the absorption trials. However, this did result in a striking improvement in the absorbability of beef tallow.

DISCUSSION

It was not possible, by using apparent absorption coefficient values, to compare the amount of fat excreted by birds fed high and low fat diets in experiments 1 and 2. Data in table 2 illustrate that the apparent absorbability of the fat in the basal diet, which contained approximately 3% of fat, was much lower than would be expected. The reason for this value is not low absorbability of corn and soybean oils, but is due mainly to the excretion of endogenous fecal (and urine) fat. A correction for endogenous and basal unabsorbed fat was not possible, since the amount of fat in the basal components of the diet was not constant at each level of added fat. Carver et al. ('55) used "total fatty acids excreted per 100 grams of feed eaten" to illustrate differences of fat utilization between high- and low-fat diets. This method has been used in experiments 1 and 2 of this study to show these differences. Where comparisons were made between treatments when only one fat level was used, as was done in experiments 3 and 4, apparent absorption coefficient values were very successfully used.

The absorbability of certain fats by the chick increases with age. It was postulated that if an adaptability of the gut to the fat was responsible for the increase in absorbability of beef tallow observed in experiment 1, then approximately the same large amount of fat, as shown by the very young chick, would be excreted in the feces, after the change from the low- to the high-fat diet, at either 2, 4, 6 or 8 weeks of age. However, as shown in figure 1, the amount of fat excreted, when the diets were so changed, decreased as age increased, and approximated the amount excreted by the group which received the high beef tallow diet continuously. Either the gut adapted rapidly to the fat (within the three-day adjustment period prior to collection), or the adaptability factors mentioned previously were not the reason for the increase in fat absorbability. More evidence which points to the latter alternative is provided by the group switched from the 20% beef tallow diet to the 10% beef tallow diet at 6 weeks of age. If adaptability factors were increased by feeding a diet high in fat, then simply reducing the quantity of fat fed should cause an increase in the utilization of that fat. However, this was not the case, and again the absorbability appeared to be related primarily to age.

The amount of fat excreted at one week of age, when 20% of beef tallow was fed, was very large. This was quickly reduced by the second week of age and proceeded to gradually decline. This indicates that the absorptive mechanism for certain fats in the very young bird is not well developed. Since safflower oil and corn oil are well utilized at this young age, it appears that the physiological or chemical processes involved in their digestion or absorption is, indeed, different from those involved with beef tallow.

It is possible that the exogenous ox bile, which would be in solution in the gut, has two functions. It may aid directly in fat absorption and/or it may stimulate the liver cells to secrete more bile acids which would aid in fat absorption. The gallbladder size of birds which received the higher ox bile levels was increased over that of the control birds. This evidence suggests that, although the bile acids present in the ox bile were not characteristic of the birds' bile acids (Deuel, '55), they were probably absorbed and had a choleretic effect on the liver.

The addition of bile caused a more dramatic fat absorption response in the pre-

liminary experiment (table 2) than in the comprehensive experiment (table 3). Unfortunately, another batch of beef tallow, showing fairly good absorbability, was used in the comprehensive experiment. This variability in tallows is important and requires further consideration.

The results of experiment 4, which tested the effect of restricted feed intake on the absorbability of fat, indicate that absorbability of fat is not dependent upon growth rate. Birds which were fed 80 and 60% ad libitum diets had much lower body weights than the controls but did not differ from the controls in their ability to utilize beef tallow.

Cheng et al. ('49) found that dietary calcium had a marked influence on the absorbability of fat in the rat. Our results clearly illustrate the same phenomenon with the chick. It is postulated that with a decrease in the ionized calcium level in the gut, a decrease in the formation of insoluble soaps occurred and more of the tallow could then be utilized.

Thus, in this paper, two factors, namely, bile sufficiency and level of ionized calcium, are shown separately to affect the absorbability of beef tallow. Whether their effects would be additive was not determined. Further work will be required to determine the relative importance of each in the age adaptation effect noted earlier.

Generally, results in experiment 1 indicate that the dietary level of fat (10 or 20%) does not alter utilization, in agreement with the results of Hoagland and Snider ('41) using rats, and thus one might assume that bile could not be limiting at the higher fat level. However, the utilization observation is a result of a combination of factors which may produce opposite effects on absorbability at various fat levels. For example, whereas a high-fat level would be expected to compete more severely for a limiting amount of bile than a lower fat level, it would have relatively less interference from the existing level of calcium and magnesium ions.

SUMMARY

The apparent absorption coefficients of fats by chicks were similar for either 10 or 20% dietary-fat levels and were high for safflower oil, corn oil and hog grease, and low for beef tallow.

The apparent absorbability of beef tallow (a) increased from 53% at one week of age to 80% at 12 weeks of age, (b) was increased when 0.5% or more of dietary ox bile was added, (c) was not dependent upon growth rate or feed intake and (d) was decreased by high dietary calcium and increased by reducing the calcium intake.

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Comparison of Effects of Palmitic and Stearic Acids in the Diet on Serum Cholesterol in Man[1]

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EXPERIMENTS IN SEVERAL laboratories showed that, in man in caloric equilibrium, isocaloric substitution in the diet of glycerides of long-chain (12 carbon and more) fatty acids for mixed carbohydrates, monoene glycerides, or polyunsaturated glycerides caused an elevation of serum cholesterol concentration (1–3). Substitution of 1% of the total calorie intake of saturated glycerides for mixed carbohydrates or monoene glycerides produced an average increase in serum cholesterol concentration of the order of 2.7 mg/dl.[4] Similarly, substitution of 1% of the total calorie intake as saturated glycerides for polyunsaturated fatty acid glycerides produced an elevation of the order of 4.0 mg/dl. On the other hand, as expected from the preceding data, isocaloric substitution of polyunsaturated fatty acid glycerides for mixed dietary carbohydrates or monoene fatty acid glycerides caused a decrease in serum cholesterol concentration of the order of 1.3 mg/dl for each 1% of the total calorie intake (3). Thus, taking as reference the serum cholesterol level of the diet containing carbohydrates or monoene triglycerides, isocaloric substitution of long-chain saturated fatty acid glycerides produces an elevation of serum cholesterol, whereas substitution of polyunsaturated glycerides decreases the cholesterol level.

Several reports in the literature (1, 4, 5), as well as previous work from this laboratory (6), indicate that, in the diet of man, cocoa butter, a fat rich in stearic acid, lacks the cholesterol-raising effect expected from its total content of long-chain saturated fatty acids. These data are consistent with the hypothesis that dietary stearic acid in contrast to other long-chain saturated fatty acids has no effect on serum cholesterol concentration in man (6). A similar view has been expressed by Hegsted et al. (2). More recently we have reported that the effect on serum cholesterol of mixtures containing cocoa butter and coconut oil can be explained mainly by the coconut oil component of the mixture (7), thus confirming that cocoa butter lacks the cholesterol-raising effect expected from its content of 18 carbon saturated fatty acids.

Although these results support the hypothesis that stearic acid in ingested cocoa butter is devoid of effect upon serum cholesterol concentration in man, it could be argued that some unknown factor in cocoa butter might be responsible for its relative lack of cholesterol-raising effect. Accordingly, a critical experiment was designed to test this question. The data to be reported offer a comparison of the effects on the serum lipids of two pairs of

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[4] dl = Deciliter = 100 ml.

diets. The first pair included cocoa butter and an artificial randomized triglyceride mixture having the same fatty acid composition. The second pair included natural palm oil, which is rich in palmitic acid, and an artificial mixture matching palm oil in regard to the distribution of the main groups of fatty acids but containing a certain proportion of stearic acid in place of palmitic acid.

METHODS

Subjects

The subjects of the experiment were 32 middle-aged (40–65 years) mentally retarded male inmates of the Faribault State School and Hospital, Faribault, Minnesota, selected after physical examination and investigation to exclude men with signs of metabolic disease. They were divided into four groups matched as to age, relative body weight, and serum cholesterol concentration. All of the men were in good physical condition and ate well at the start. Later during the course of the experiment two of the men suffered acute illness and lost weight. For this reason their data were excluded. Accordingly, two groups had eight subjects each, and the other two groups had seven subjects.

Diets

There were four experimental diets, made up of a single basic low fat diet and one of four fat supplements, each providing 86 g fat daily. The fat supplements were incorporated into certain menu items, such as, hot breakfast cereal, mashed potatoes, and casserole dishes. The diet was made up as 14 different daily menus that were served in rotation and comprised foods usually found in American diets including lean meat, fish, or cheese twice a day. The energy value of the standard daily serving was 2,480 kcal, of which 1,705 kcal were provided by the basic diet and 775 kcal by the fat supplement. Total food intake was adjusted individually in order to maintain constant body weight, which was measured twice a week, the adjustment being made by adding or removing bread and jelly. A record was kept of the food rejected by each individual for each day of the experiment. Standard servings of the daily diet were pooled for the last 14 days of each dietary period and analyzed by methods previously described (8). The average food intake for all of the individuals in each dietary period was calculated from the analysis of the pooled standard servings and the record of rejections and supplements. The calorie value and nutrient content of the rejections and adjustments were calculated from standard food tables (9).

The test fat supplements were prepared as follows. *Supplement 94CB* was made by mixing 94 parts of cocoa butter with 6 parts of safflower oil. *Supplement ICB* (imitation cocoa butter), intended to match the *94CB* supplement in fatty acid composition, was prepared by mixing 42.0 parts of palm oil, 34.4 parts of totally hydrogenated soybean oil, 23.0 parts of olive oil, and 0.6 part of safflower oil. *Supplement PO* consisted of natural palm oil. *Supplement IPO* (imitation palm oil) was intended to match palm oil in the distribution of the three main groups of fatty acids but to have more stearic acid and less palmitic acid than natural palm oil. It was prepared by mixing 47.3 parts of totally hydrogenated soybean oil, 45.5 parts of olive oil, and 7.2 parts of safflower oil. Both supplements, *ICB* and *IPO*, were subjected to randomization with 0.25% NaK catalyst followed by deodorization in the Miami Valley Research Laboratory of the Procter and Gamble Company, Cincinnati, Ohio. The data kindly provided by Dr. Mattson indicate that 100 g of *mixture ICB* contained 19 g trisaturated triglycerides including 3 g triglycerides made up only of 18- or 20-carbon atom saturated fatty acids. *Supplement IPO* contained, per 100 g, 14 g trisaturated triglycerides including 8 g triglycerides made up only of 18- and 20-carbon atom saturated fatty acids.

The four experimental diets were fed simultaneously for periods of 18 days to each of the four experimental groups of men. The diets were changed in successive dietary periods in a symmetrical Latin-square design so that by the end of the experiment each man had eaten each of the diets and each group had eaten the diets in a different order. The actual intake of energy and nutrients and the glyceride distribution of the average daily food eaten per man on each of the four experimental diets are presented in Table 1. Each experimental diet is designated by the name of its fat supplement. It is apparent from the table that, except in regard

TABLE 1

Caloric value and nutrient intake of the food eaten daily (means for 30 men)

Item	Diets			
	94CB[a]	*ICB*[b]	*PO*[c]	*IPO*[d]
Energy, kcal	2,750	2,740	2,800	2,710
Protein, g	97	97	99	96
Carbohydrates, g	353	351	360	345
Total lipid, g	105	105	106	105
Test fat supplement, g	83	83	84	82
P third of phospholipids, g[e]	0.6	0.5	0.7	0.5
Unsaponifiable lipid, g	2.0	2.0	1.6	2.1
Cholesterol, mg	145	144	146	144
Glycerides, g[f]				
Total	102	102	103	102
Saturated				
<12-C	1	1	1	1
12 to 16-C	27	28	46	15
>16-C	31	30	7	38
Total saturated	59	59	54	54
Monoene	35	35	37	37
Polyene	8	8	12	11

[a] Experimental fat, 94% cocoa butter, 6% safflower oil. [b] Experimental fat, mixture matching fatty acid composition of *94CB*. [c] Experimental fat, palm oil. [d] Experimental fat, mixture matching fatty composition of palm oil except for containing more stearic and less palmitic acids. [e] Lipid P × 6.3. [f] Glycerides = total lipid − P third of phospholipids − unsaponifiable lipid.

to particular fatty acids, the four diets match each other and that the actual intakes of the different constituents were practically the same for the different dietary periods.

All of the diets were comparable in the amount of saturated fatty acids with fewer than 12 carbon atoms and in the amount of monoenic fatty acids. *Diets PO* and *IPO* contained slightly more polyene fatty acids than *diets 94CB* and *ICB*, but polyenes provided only a very small proportion of total calories, 2.7 and 2.8% in *diets 94CB* and *ICB* and 3.8 and 3.6% in *diets PO* and *IPO*, respectively. On the other hand, *diets PO* and *IPO* contained slightly less total saturated fatty acid than *diets 94CB* and *ICB*, but the difference amounted to only 45 kcal/day. *Diets 94CB* and *ICB* were practically identical in saturated fatty acids with 12- to 16-carbon atoms and in saturated fatty acids with more than 16 carbon atoms in the chain. In contrast, *diet PO* contained two-thirds more 12- to 16-C saturated and less than one-fourth of the longer chain saturated fatty acids provided in *diets 94CB* and *ICB*. On the other hand, *diet IPO* contained only a little more than half the 12- to 16-C saturated fatty acids, but one-fourth more long-chain saturated fatty acids than provided in *diets 94CB* and *ICB*. In these respects there was a great difference between *diets PO* and *IPO*—three times as much 12- to 16-C saturated fatty acid in *PO* as in *IPO* and five times as much longer chain saturated fatty acids in *IPO* as in *PO*.

The daily portion of each of the four test fat supplements contained less than 0.5 g lauric acid and, except for *test fat PO*, an equally small amount of myristic acid. *Test fat PO* contained about 1 g myristic acid as glycerides daily. The amount of lauric and myristic acids in the basic diet was small and constant. It is clear that the differences between diets in the glyceride category labeled saturated 12- to 16-C were practically all due to palmitic acid. *Diet PO* provided daily about 31 g more of palmitic acid than did *diet IPO*. Similar considerations show that the differences in the category saturated > 16-C were almost exclusively glycerides of stearic acid. *Diet IPO* provided about 31 g more of stearic acid than *diet PO* did daily.

Serum Lipid Analyses

Fasting venous blood samples were taken at the end of each of the four 18-day dietary periods. The serum was analyzed for cholesterol and lipid phosphorus by methods previously described (10) and for triglycerides as described by Pantulu et al. (11). Statistical analysis was performed taking into account the use of a symmetrical Latin square in the change of diet in successive periods. From the analysis of variance (12), *t* tests were calculated for the comparisons of interest. Control of the significance level for the multiple *t* tests was carried out by Tukey's procedure (13).

Stool Analyses

An attempt was made to get stool samples from subjects selected for their ability to cooperate. Weighed chromium oxide tablets were given before each meal as a time index. Unfortunately, the patients proved less capable of cooperation than expected and adequate stool collections were achieved only with four subjects in two dietary periods. Homogenates of the stool composites were analyzed for chromium and for total fatty acids. Chromium was determined by ashing and fusing with sodium peroxide as described by Schürch et al. (14) followed by development of a colored compound of chromate with 1,5-diphenylcarbohydrazide as described by Allen (15). Fecal fat was determined by the method of Van de Kamer et al. (16).

RESULTS

The body weights of the 30 subjects whose data were used remained within the predetermined ranges of 2 kg/period and 3 kg in the whole experiment. The mean weights at the time of each blood sampling are given in Table II. The constancy of body weight is considered to insure that changes in blood lipid concentrations were not due to changes of energy balance.

The mean concentrations of cholesterol, phospholipids, and triglycerides in serum are given in Table III for each group of men in each period.

The mean serum lipid values for each of the four dietary situations for all 30 men are presented in Table IV. The serum cholesterol and phospholipids were practically identical when the men ate *diets 94CB* and *ICB*. Statistical analysis demonstrated no significant difference between these values. Serum triglyceride levels were on the average 12 mg/dl higher at the end of the dietary period with *ICB*, but this difference was not statistically significant ($P = 0.14$).

Comparison of the lipid values for the other pair of diets shows that *diet PO* produced higher serum cholesterol and phospholipids but lower triglycerides than *diet IPO*. The serum lipid differences between these two dietary periods were all statistically significant as shown by the data presented in Table V.

TABLE II
Mean body weight at the end of each dietary period

Group	Number of subjects	Diets			
		94CB, kg	*ICB*, kg	*PO*, kg	*IPO*, kg
W	7	64.5	64.3	64.5	64.7
X	8	67.4	67.7	67.5	67.4
Y	8	69.8	70.3	70.6	70.2
Z	7	66.8	66.2	67.0	67.0
All	30	67.2	67.5	67.5	67.5

All possible differences between the four dietary treatments for each of the three serum lipid fractions were examined by analysis of variance. Between-diet differences are significant at the 0.01 probability level if they are greater than the following critical values (mg/dl): 9 for serum cholesterol, 8 for serum phospholipids, and 20 for serum triglycerides. No difference failing to reach the significance level of $P = 0.01$ proved to be significant at the 0.05 level. *Diet PO* produced significantly higher serum cholesterol levels than *diets ICB* and *94CB*, the differences being, respectively, 18 and 14 mg/dl. Likewise, *diet 94CB* produced serum cholesterol significantly higher (10 mg/dl) than *diet IPO*. On the other hand, there was no significant serum cholesterol difference between *diets IPO* and *ICB*.

Diet PO caused significantly higher serum phospholipid levels than *diets ICB* and *94CB* (11 and 10 mg/dl, respectively). However, no significant difference in serum phospholipids was found between *diets IPO* and *ICB* or *IPO* and *94CB*.

A significant difference in serum triglyceride concentration (21 mg/dl) was observed between *diets ICB* and *PO*. *Diet*

TABLE III

Mean serum lipid concentrations, mg/dl, for each group at the end of each period

Group	Number of subjects		Periods 1	2	3	4
W	7	Diet*	*PO*	*IPO*	*ICB*	*94CB*
		Cholesterol	233	198	220	209
		Phospholipids	227	210	219	211
		Triglycerides	121	160	131	125
X	8	Diet*	*IPO*	*PO*	*94CB*	*ICB*
		Cholesterol	184	203	196	186
		Phospholipids	186	204	198	195
		Triglycerides	88	77	91	94
Y	8	Diet*	*94CB*	*ICB*	*IPO*	*PO*
		Cholesterol	200	187	196	212
		Phospholipids	197	198	202	212
		Triglycerides	96	121	114	104
Z	7	Diet*	*ICB*	*94CB*	*PO*	*IPO*
		Cholesterol	205	208	223	197
		Phospholipids	208	218	220	211
		Triglycerides	144	133	104	126

* See footnotes in Table I.

TABLE IV

Serum lipid concentrations, means (±SD) for 30 men, mg/dl

Diet*	Total cholesterol	Phospholipids	Triglycerides
94CB	203 ± 29	205 ± 24	110 ± 32
ICB	199 ± 29	204 ± 22	122 ± 54
PO	217 ± 34	215 ± 26	101 ± 40
IPO	193 ± 31	202 ± 26	121 ± 51

* See footnotes in Table I.

ICB gave the highest serum triglyceride levels. *Diet IPO* gave the second highest triglyceride levels and the lowest total cholesterol and phospholipid. The other serum triglyceride differences did not reach statistical significance.

For the interpretation of the results it is important to consider the fatty acid composition of the two diets involved in each of the two main dietary comparisons. Table VI presents the percent contribution to the total calorie intake of the various categories of fatty acid glycerides for *diets 94CB* and *ICB*. It is apparent that the two diets were well matched in terms of their fatty acid composition. These diets gave practically equal serum lipid levels.

Table VII presents the corresponding data for *diets PO* and *IPO* showing that *diet PO* had a content of 12- to 16-carbon fatty acid glycerides (mainly palmitic acid)

TABLE V

Serum lipid differences associated with replacing 10% of the total calorie intake of stearic acid glycerides by palmitic acid glycerides

	Differences, *PO* minus *IPO*, mg/dl		
	Mean	SE	*P*
Cholesterol	24	3.1	<0.0001
Phospholipids	13	3.0	0.0001
Triglycerides	−20	6.7	0.006

higher than that of *diet IPO* by 9.9% of total calorie intake. On the other hand *diet IPO* had a content of stearic acid glycerides (saturated >16-C) higher than that of *diet PO* by 10.4% of total calorie intake. These two diets gave significantly different levels of all three serum lipids.

For the study of the possible failure of absorption of fat due to the presence of high melting triglycerides, *diet IPO* was chosen because it contained the largest amount of stearic acid and also the largest proportion of trisaturated triglycerides made up exclusively of 18-C and 20-C fatty acids. The fecal fatty acids were expressed as daily excretion values by making the assumption that the quantity of fecal homogenate that contained the same amount of Cr_2O_3 as was given in 1 day was equivalent to the average daily intestinal output. The stools of one subject had high fatty acid contents with wide variations (20–46 g/day) unrelated to the type of diet. However, this subject kept constant body weight throughout the experiment and his serum lipid changes were similar to those observed in the other men. Consequently, we include his data. We do not know whether there were any other individuals with similar high fecal fat excretion among the 26 who did not have stool analysis. For the other three subjects from whom feces were obtained, the mean of fecal fatty acids when they were receiving *diet 94CB* was between 5 and 8 g daily. When shifted to *diet IPO*, the fatty acid excretion increased by about 4 g daily. The corresponding quantities of trisaturated triglycerides in the amounts of test fat supplements of the two diets actually eaten (see Table I) were 2 g in *94CB* and 12 g in *IPO*.

TABLE VI

Glyceride composition of diets eaten, % of total energy

Fatty acid glyceride class	*94CB*[a]	*ICB*[b]	*94CB* − *ICB*
Saturated <12-C	0.3	0.3	0
Saturated 12 to 16-C	8.8	9.2	−0.4
Saturated >16-C	10.1	9.9	0.2
Total saturated	19.2	19.4	−0.2
Monoene	11.5	11.4	0.1
Polyene	2.7	2.8	−0.1

[a] *94CB* = diet containing the fat supplement made of 94% cocoa butter and 6% safflower oil.
[b] *ICB* = diet containing as fat supplement a randomized mixture of 42% palm oil, 23% olive oil, 34.4% totally hydrogenated soybean oil, and 0.6% safflower oil.

TABLE VII

Glyceride composition of diets eaten, % of total energy

Fatty acid glyceride class	*PO*[a]	*IPO*[b]	*PO* − *IPO*
Saturated <12-C	0.3	0.2	0.1
Saturated 12- to 16-C	14.7	4.8	9.9
Saturated >16-C	2.4	12.8	−10.4
Total saturated	17.4	17.8	−0.4
Monoene	12.1	12.5	−0.4
Polyene	3.8	3.6	0.2

[a] *PO* = diet containing the palm oil supplement.
[b] *IPO* = diet containing as fat supplement a randomized mixture of 47.3% totally hydrogenated soybean oil, 45.5% olive oil, and 7.2% safflower oil. The basic difference between these two diets is that in *diet PO* palmitic acid is higher and stearic acid is lower, each by about 10% of calories.

DISCUSSION

The results reported indicate that the diets containing natural cocoa butter (*94CB*) and imitation cocoa butter (*ICB*) produced similar levels of the three serum lipid fractions studied. The nutrient content of the diets, including the various categories of fatty acids, were practically identical. The diets differed, however, in the distribution and location of fatty acid residues within triglyceride molecules. Cocoa butter is characterized by having about half of its glycerides as oleopalmitostearin and only 2–3% as fully saturated

triglycerides (17). Its melting point is between 28 and 36 C (18). The matching randomized mixture (*ICB*) according to Dr. F. Mattson's calculations had 19% of trisaturated triglycerides corresponding to a solids content of 19% at 40.5 C. This indicates that these two fats with similar fatty acid composition are associated with essentially similar serum lipid levels in spite of the fact that the glyceride structures are evidently different. It further indicates, in agreement with our previous suggestion (6), that neither the glyceride structure of cocoa butter nor the possible existence of a cholesterol-depressing substance in that fat explains its effect on serum lipid levels in man.

The structural differences between these two fats suggest the possibility that they may have been differently absorbed in the intestine. From the content of trisaturated triglycerides mentioned above it can be calculated that the cocoa butter-containing diet (*94CB*) provided about 2 g/day of these glycerides, whereas the diet containing the mixture imitating cocoa butter (*ICB*) had about 16 g/day of them. If the trisaturated triglycerides were completely unabsorbed there would be a difference of 14 g in the amount of fat absorbed between these two diets. However, as indicated in Table II, the body weights of the subjects were practically constant throughout the whole experiment, and this together with the fact that the food intake was identical for these two diets may be taken as an indication that the fats were equally well absorbed. The coefficient of digestibility of cocoa butter in man has been reported by Deuel (18) to be 95%, which is within the range of values (94–98%) reported in the same place for almost all food fats. Consequently, it appears that both fats were well absorbed and that the failure of cocoa butter to raise serum cholesterol cannot be explained by failure of absorption of its saturated fatty acids. The limited number of analyses of fecal fat performed in three subjects indicate that the apparent absorption of total dietary fat when the men were eating cocoa butter was of the order of 94%.

On the other hand the comparison between the *diets PO* and *IPO* clearly indicates that the replacement of palmitic acid by stearic is associated with a decrease in serum cholesterol and phospholipid concentrations. This is also in agreement with our previous suggestion that the effect of cocoa butter on man's serum lipids can be explained by assuming that stearic acid glycerides are devoid of any cholesterol-raising effect. Under this assumption, using the equation of Fig. 1 of our previous publication (6), we have calculated that the serum cholesterol difference expected when *diet IPO* replaces *diet PO* is 24 mg/dl, the same as the observed mean difference.

This computation is equivalent to saying that isocaloric replacement of carbohydrates by 31 g palmitic acid glycerides daily will cause an increase of serum cholesterol concentration of 24 mg/dl. Since the serum cholesterol concentration when the men were eating the *IPO* diet was exactly 24 mg/dl lower than that observed when they ate *diet PO*, it follows that stearic acid caused serum cholesterol concentration identical to that on a diet with an isocaloric amount of carbohydrate. Accordingly, we interpret this to indicate that dietary stearic acid has no cholesterol-raising effect as compared with dietary mixed carbohydrates. The effect observed is the same as if the 12- to 16-C saturated glycerides had been isocalorically replaced by carbohydrate or by monoene glycerides, the foodstuffs that by definition are taken to have zero cholesterol-raising effect.

The lack of effect of >16-C saturated glycerides may depend in part on incomplete absorption, but it must be pointed out that the effect of dietary factors on

serum cholesterol concentration may sometimes not be dependent on their absorption from the intestine but simply on their presence in the intestinal contents. For example, beta-sitosterol is known to have a serum cholesterol-depressing effect in spite of the fact that it is poorly absorbed (19). Conversely, the small cholesterol-raising effect of cocoa butter can not be attributed to lack of absorption because, as already mentioned, cocoa butter is well absorbed in man (18).

Because tristearin is known to be poorly absorbed, it seems useful to make an estimate of unabsorbed fat by assuming that none of the dietary tristearin was absorbed. The melting-range data of *mixture IPO* indicates that it had a tristearin content of 8%, which corresponds to 7 g tristearin/man per day. *Supplement PO* did not contain any appreciable amount of tristearin. *Diet IPO* contained 31 g more per day of total stearic acid glycerides as compared with *diet PO*. In other words, the replacement of *diet PO* by *diet IPO* under this assumption will be equivalent to removing 31 g palmitic acid glycerides from *diet PO* and replacing it by 24 g (i.e., 31 − 7) stearic acid glycerides. Using the same system of calculation as before, it is found that if the amount of stearic acid likely to have been absorbed had the same cholesterol-raising effect as palmitic acid, the serum cholesterol concentration would have decreased by 5 mg/dl instead of the observed decrease of 24. This limiting calculation indicates that taking into account the possibility of incomplete absorption of stearic acid does not explain the difference in serum cholesterol levels between *diets PO* and *IPO*. Moreover, the constancy of body weight and the small difference observed in fecal fat indicate that failure of absorption was not the explanation of the low serum cholesterol levels of the men when eating the diets containing stearic acid. The results support, therefore, our previous suggestion regarding the lack of effect of dietary stearic acid on serum cholesterol concentration (6).

This concept helps to explain the lack of effect of stearic acid reported by Ahrens et al. (1) and Horlick and Craig (20) that we discussed in our previous communication (6).

The fact that *diet PO*, providing 10% excess of the calorie intake as palmitic acid glycerides, produced a higher serum cholesterol level than *diet IPO*, having a similar excess of stearic acid glycerides, clearly indicates that, relative to stearic acid, palmitic acid has a cholesterol-raising effect. Furthermore, the observed serum cholesterol difference between *diets PO* and *IPO* agrees very well with that predicted by the equation derived from experiments including mixtures containing significant amounts of lauric and palmitic acids as well as small amounts of myristic acid. This could be taken as an indication that there is no great difference between the effects of each of these fatty acids. This is also supported by the statement by Hegsted et al. (2), who found no difference in a direct comparison between palmitic and myristic acids in the presence of safflower oil. Therefore, the present experiment supports our previous suggestion that the cholesterol-raising effect of saturated fatty acids in natural human diets is due to lauric, myristic, and palmitic acids. Palmitic acid is much more abundant than lauric or myristic in most of the fats present in the usual Western diets. Accordingly, palmitic acid glycerides must be considered responsible for most of the cholesterol-raising effect of such diets. In the present experiment, *diet PO*, with the highest content of palmitic acid and lowest of stearic, gave the highest serum cholesterol level and *diet IPO*, with the lowest palmitic acid and highest stearic, gave the lowest serum cholesterol level. *Diets 94CB* and *ICB* with intermediate composition of saturated fatty acids gave

intermediate levels of serum cholesterol. The serum phospholipid differences were roughly parallel to those of cholesterol.

Serum triglycerides tended to change in the direction opposite to the changes of serum cholesterol and phospholipids. *Diet PO* gave the lowest serum triglyceride level. The differences between *diet PO* and *diets ICB* and *IPO* were significant, but not that between *diets PO* and *94CB*. No other possible serum triglyceride difference reached statistical significance.

SUMMARY

Previous observations have indicated that stearic acid, in the diet of man, lacks the cholesterol-raising effect of other saturated fatty acids. A critical test of the lack of effect was made by comparing cocoa butter, the fat used in the original experiments, with a mixture of other fats matching cocoa butter in linoleic, oleic, palmitic, and stearic acids. These fatty acids were provided mainly by safflower oil, olive oil, palm oil, and totally hydrogenated soybean oil, respectively. The mixed fat was randomized to improve digestibility. Serum lipid levels of 30 men were identical when cocoa butter and the mixed fat were fed alternately for 18-day periods. This result shows that cocoa butter does not contain any special cholesterol-depressing substance. A second comparison was made between palm oil and a randomized fat mixture of the same four constituents. This mixture was so prepared as to match palm oil in its content of linoleic, oleic, and total saturated fatty acids. The mixture, however, provided daily 31 g stearic acid (10% of energy) more than palm oil, whereas palm oil provided 31 g more of palmitic acid daily. Serum cholesterol and serum phospholipids were higher by 24 and 13 mg/dl, respectively, when the men were eating the palm oil diet. Serum triglycerides were lower by 20 mg/dl. All these differences were statistically significant. The observed serum cholesterol difference agreed with that predicted, assuming that stearic acid, like oleic acid or mixed carbohydrate, has no effect on serum cholesterol concentration in man.

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CHAPTER 9

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Effects of Specific Fatty Acids on Serum Cholesterol in Man: Studies with Semisynthetic Materials [1]

INTRODUCTION

The studies to be reported were directed largely toward the determination of the specific role played by different fatty acids in regulating the serum cholesterol level in man. We believe that the experiments have certain special features which make them relatively unique and of special merit. The data were obtained over a one and one-half year period with essentially the same group of men. Of the 21 men commencing these studies, 3 were removed; 18 men were present in all periods. Each test diet has been fed to 9 to 11 men over a 4-week period. At intervals all of the men received the so-called control diet. All changes in serum cholesterol were compared to the mean level of serum cholesterol of the same man during the control period immediately preceding and following the test diet. Fortunately, the mean serum cholesterol levels while on the control diet have been essentially the same and we feel that the procedure used makes the data obtained over a rather long period directly comparable. Difference between men are controlled by always comparing the same individuals.

The men were middle-aged mental patients, mostly chronic schizophrenics, who had no apparent physical defects. All individuals with serum cholesterol levels above 300 mg % or below 200 mg % were deliberately excluded from the study. The mean serum cholesterol levels when the men were consuming the control diet which approximates the average American

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These studies were made possible by the cooperation of several individuals and organizations. The facilities and subjects at the Danvers State Hospital were made available by Dr. Peter B. Hagopian, Superintendent. The fats and oils used were supplied by Dr. Fred H. Mattson, Miami Valley Laboratories, Procter and Gamble Co., Cincinnati, Ohio, and the milk and ice cream were supplied through the courtesy of Dr. H. L. Wilhasin and Dr. C. H. Pyne of the Hood Milk Co., Boston, Mass.

We gratefully acknowledge assistance from the Department of Biostatistics, Harvard School of Public Health. Mr. Raymond Neff developed the computer program for these analyses and Dr. Jane Worcester assisted in the interpretation of the results.

Laboratory assistance was supplied by Miss Rita Antonellis and Miss Tung-in Wang. Supervision of the meals and food preparation was performed by Miss Paula Morris, Miss Maureen McKenna, and Mrs. Paula Dolan.

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diet in composition, was approximately 220 mg %. Thus, there is reason to believe that the responses obtained will approximate those which may be found in average American men.

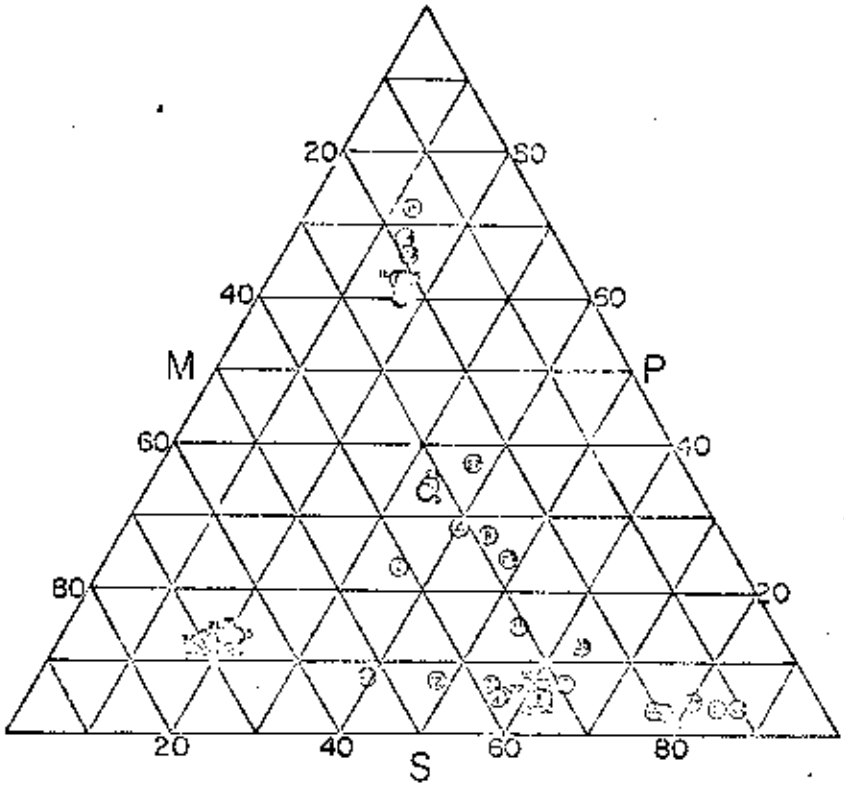
Also, the diets approximate those which would be useful in feeding the American public. A variety of low-fat foods were selected, including lean meat and about one egg per day, and to these the test oils were added. This was done by the preparation of filled milk and ice cream, by baking pies, cakes, muffins, waffles, etc. containing the experimental oils, and by use of the oils in salad dressings and spreads. Much effort was expended in developing acceptable foods. Any dietary factors other than fat which may influence serum cholesterol levels in ordinary diets should be present in both the control period and the experimental period diets.

Finally, with essentially this same group of men, we have studied a larger variety of fats and oils than have been tested under standardized conditions. These include substantial differences in all of the major fatty acids which occur in edible fats and oils. In the earlier work natural materials and mixtures of them were used. Since these materials do not provide all of the variations desired, the latter work has been done with semisynthetic materials. These were prepared by mixing specific triglycerides with another oil and then preparing the mixed triglycerides by transesterification. This was necessary since some of the saturated triglycerides are not well digested and absorbed.

Previous Studies

I should like to briefly review the results obtained during the early studies on essentially this same group of subjects which have already been published (Hegsted *et al.* 1965; McGandy *et al.* 1966). In the first paper the results of some 36 different tests were described. These trials included various natural oils and mixtures of oils which were fed at either 38 or 22% of the calories. The 22% level was selected as approximately the lowest level of fat which can be utilized and still produce reasonably palatable diets. Dietary cholesterol was also varied by removing eggs or adding additional eggs to the diet. In these studies we relied heavily upon safflower oil, olive oil and coconut oil because these oils have the largest amounts of polunsaturated (P), monounsaturated (M), and saturated (S) fatty acids, respectively, that can be found in readily available oils. Thus, maximum changes in S, M, and P could be obtained (Fig. 49).

The results of the studies were evaluated by multiple regression analysis attempting to determine the relative importance of various fatty acids in raising or lowering serum cholesterol levels. The major conclusions from these data were as follows:



From Hegsted et al. (1965)

Fig. 49. Triangular plot showing composition of oils tested in earlier studies

(1) The only variables which we could identify which clearly influenced the level of serum cholesterol were myristic acid, palmitic acid, polyunsaturated fatty acid (linoleic acid), and dietary cholesterol. Figure 50 shows the best prediction equation when the fatty acids in the diet are expressed as per cent of dietary calories. There was particular interest in this equation since myristic acid had a coefficient approximately four times as large as palmitic acid. These coefficients represent the change in serum cholesterol in mg % apparently induced by an increase of one per cent of calories as the specific fatty acid.

(2) Dietary cholesterol was also shown to be a significant factor as most investigators have now concluded. The regression equation in Fig. 50 indicates that an increase in dietary cholesterol of 100 mg results in an increase in the serum cholesterol of approximately 5.5 mg %. Furthermore,

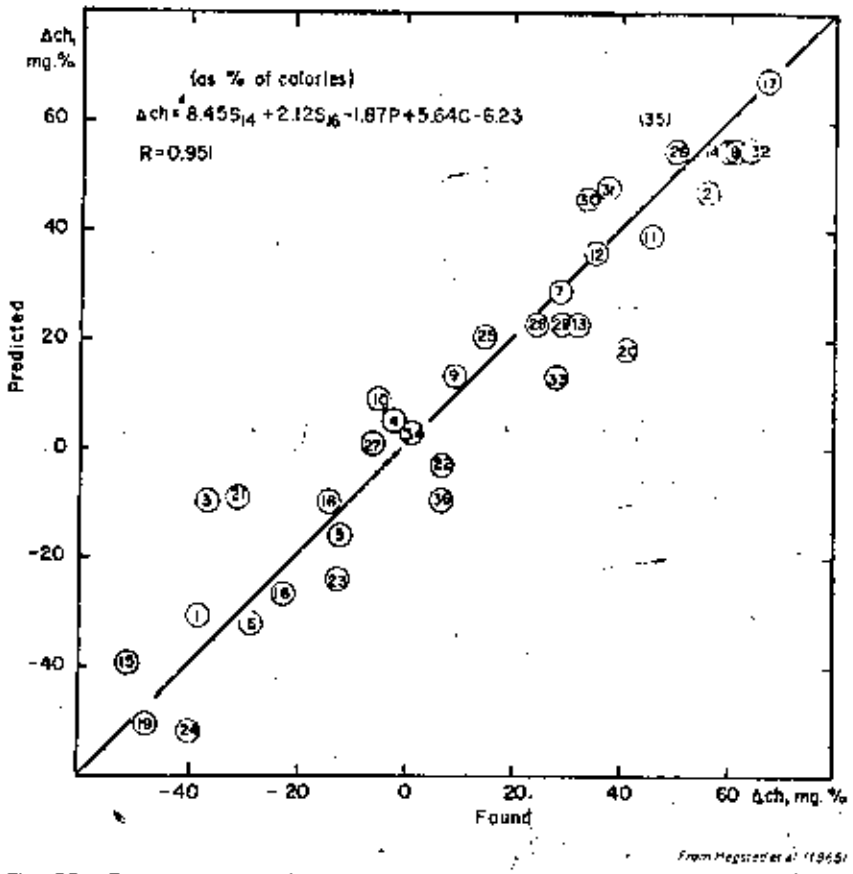
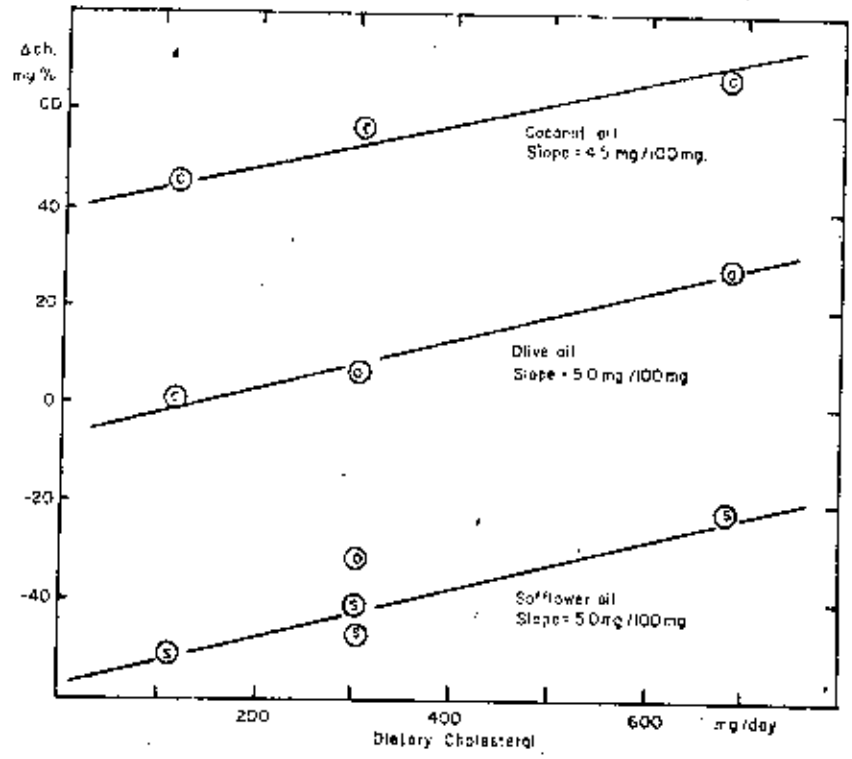


Fig. 50. Scatter diagram showing correlation between Δ cholesterol values found and predicted by the regression equation

when cholesterol was varied in the presence of different fats (Fig. 51), the response seemed to be the same. This is to say, the effect of the dietary cholesterol seemed to be independent of the kind of fat in the diet.

(3) Finally, the changes in serum cholesterol could be equally well predicted when the fatty acids were expressed not as per cent of calories but as percentage of the dietary fat (Fig. 52). In other words, as near as we can tell it was not the amount of these fatty acids in the diet that was of primary importance but rather the proportions of fatty acids present. This has practical implications since it is difficult to achieve high ratios of polyunsaturated acids to palmitic and myristic acid with low fat diets. The most effective diets are those that are high in fat.

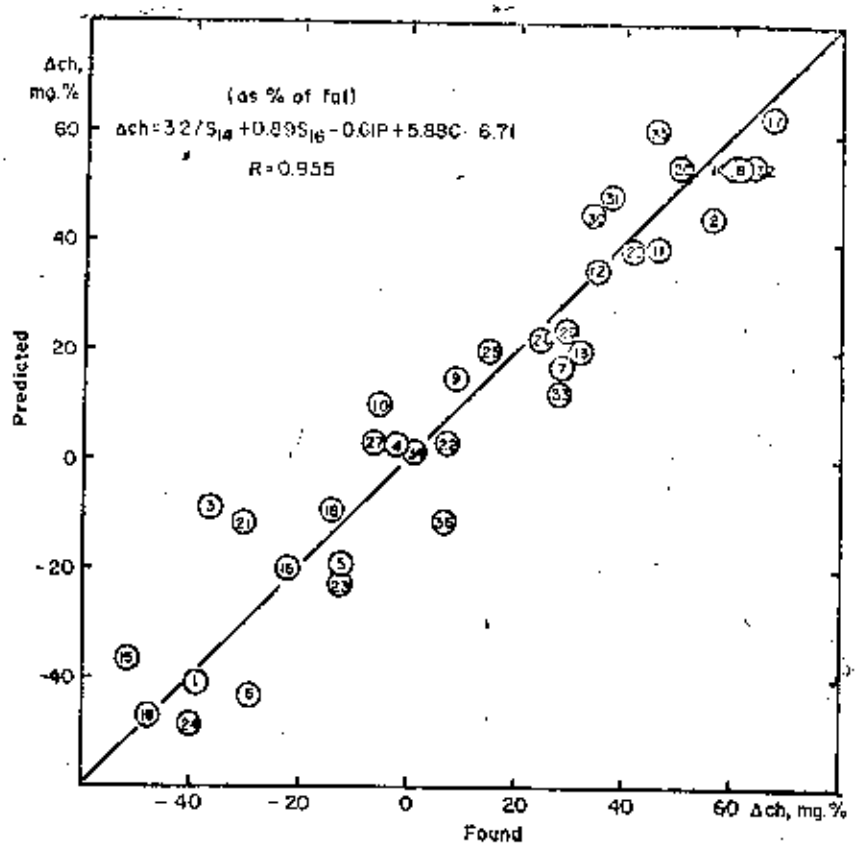


From Hegsted et al. (1965)

Fig. 51 Response of serum cholesterol to dietary cholesterol in the presence of different fats

The second study, also published, dealt with effects of carbohydrates of different kinds (McGandy *et al.* 1966). In our experience, when high-sugar or high-starch diets were compared in the presence of different oils, very little effect of the carbohydrate could be detected, and certainly nothing comparable to the large effects of fats upon serum cholesterol. It seems likely to us that if the kind of carbohydrate is significant, it is only when low-fat diets are fed. Since high-sugar low-fat diets are not an ordinary kind of diet anywhere in the world, studies with these kinds of diets have little relevance and we feel the role of carbohydrate has been overemphasized by some authors (McGandy *et al.* 1967).

In the first paper (Hegsted *et al.* 1965) of this series we emphasized some of the uncertainties. "The major difficulties of studies of this kind in arriving at firm conclusions as to the physiological role of dietary components should be clearly understood. When naturally available oils are used, the



From Hegsted et al. (1965)

Fig. 52. Scatter diagram showing correlation between Δ cholesterol values found and predicted by the regression equation

Fatty acids expressed as percentage of the dietary fat.

dietary variables are correlated to a greater or lesser extent. Much of the same information is imparted regardless of which variable is considered . . ." We are simply saying that if you raise the amount of one component in the diet, say the polyunsaturated fatty acid, something else has to change. Usually several things change. One cannot really tell whether the effect observed is due to the increase in the one component or the fall in others. We also emphasized as has Snedecor (1956) that "the size of the regression coefficients depends upon the variables included in the equation" and gave several examples of this. Thus, "These equations are primarily

descriptive of the information from which they have been derived" and "it is somewhat hazardous to attach as much functional significance to the regression equations as Keys *et al.* (1957) have done."

Recent Studies

Our primary intent in the new studies which I wish to present briefly today has been:

(1) To try to prepare and test a series of oils in which specific fatty acids could be independently varied. If it were possible, for example, to substitute myristic acid for palmitic acid and keep everything else the same, then and only then, could one be reasonably sure what the difference was between myristic and palmitic acid.

(2) We wished to obtain and test some oils in which specific fatty acids, such as myristic and stearic acid, would be fed in combinations that just could not be found in natural oils. It is true that trimyristin, tripalmitin, and tristearin are available and could thus be added independently of each other, but this did not seem advisable since tripalmitin and tristearin are known to be poorly utilized in this form. We had, therefore, to resort to triglycerides prepared by transesterification.

Even this approach has some rather serious limitations in what can be achieved in fatty acid composition. If one begins to add saturated fatty acids to olive oil, for example, one soon reaches a point where substantial amounts of trisaturated glycerides are formed. The maximum amount of any fatty acid that can be present in a triglyceride without forming triglycerides of that fatty acid is 66%. Of course, some is formed at much lower levels in randomly esterified preparations. In order to get around this constraint to some extent, we have also utilized some preparations made with medium chain triglycerides (MCT) to which saturated fatty acids were incorporated by transesterification. This was thought worthwhile since MCT itself seems to have little effect on serum cholesterol. While this latter conclusion is supported by a variety of studies, it has not been tested in the presence of a large series of oils. Also, we may note that even the statement that "it has little effect on serum cholesterol" is ambiguous since one has to compare it to something else. In our studies MCT gives about the same serum cholesterol levels that one achieves with olive oil and with our control diet. We could also say that MCT lowers the serum cholesterol when compared to coconut oil or raises the level when compared to safflower oil.

The composition of the series of oils that have been tested in recent months and the resultant changes in serum cholesterol are shown in Fig. 53 and Fig. 54, and in Table 59. The order in which they are arranged on the slide is not the order in which they were tested. Table 59 indicates the order in which they were studied. In the first series we have safflower oil

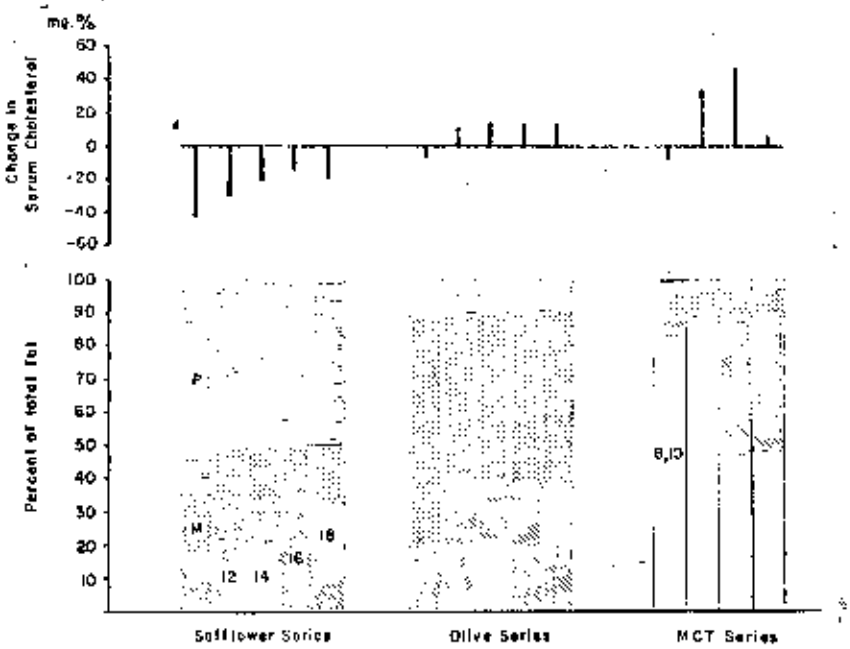


Fig 53. Percentage distribution of fatty acids in test fats and change in serum cholesterol

and safflower oil into which substantial amounts of lauric, myristic, palmitic, and stearic acid were incorporated by transesterification. In this series lauric acid appeared less hypercholesterolemic than the other saturated acids, but whether there is any difference in myristic, palmitic, and stearic acid is not clear. In the next series in which the four saturated acids were incorporated into olive oil, all appear to have about the same effect. In the third series we have MCT and MCT to which myristic, palmitic and stearic acid were incorporated. Palmitic seems to be most hypercholesterolemic, myristic acid less, and stearic acid still less.

In the fourth series these same MCT mixtures were fed with a small amount of safflower oil and in the following series with a small amount of olive oil. Here myristic acid appeared most active, with palmitic and stearic less, and perhaps about the same. Finally, when two of these MCT preparations—the myristic and stearic acid preparations—were fed, first with about half of the fat as safflower oil and then with olive oil, not much difference could be detected. For comparison the last three bars indicate the composition of the control diet, of coconut oil, and cocoa butter. The control diet is the baseline for calculating the changes in serum chol sterol.

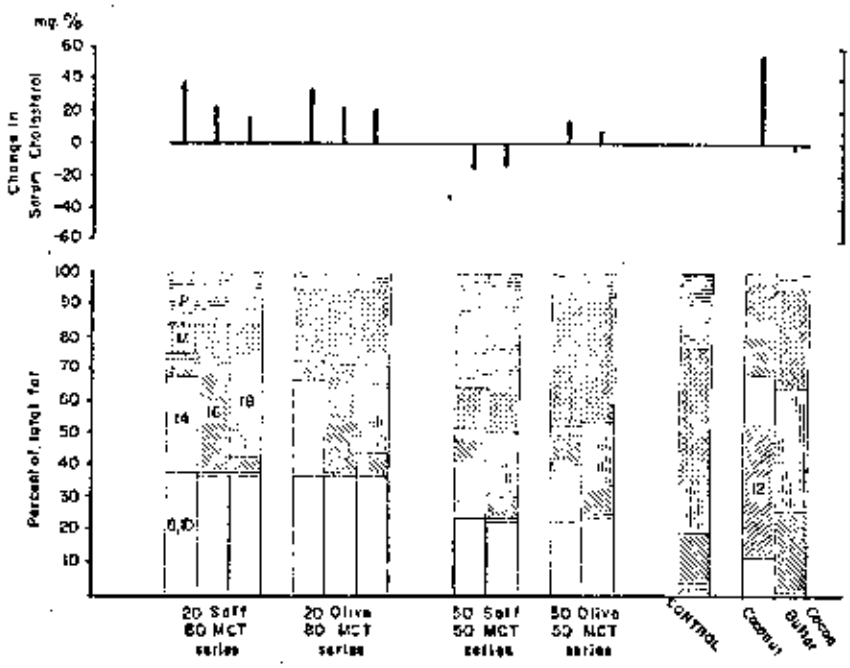


Fig. 54. Percentage distribution of fatty acids in test fats and change in serum cholesterol

It is worth emphasizing again that the total swing in serum cholesterol from +50 to about −50 mg % is very large considering that these men have "normal" or below normal levels of serum cholesterol (220 mg %) on the control diet.

Cocoa butter, which is a somewhat unique natural oil in that it is rather highly saturated but contains practically no saturated acids of less than S_{16}, does not elevate the serum cholesterol appreciably. This was noted by us earlier and by Keys *et al.* (1965A), and it suggested to both groups that stearic acid had effects different from palmitic and myristic acids.

The results of this series do not appear consistent. In one set (with large amounts of MCT), stearic acid seems much less effective than myristic and palmitic acid, in several tests slightly less effective, and in some not different from the other saturated fatty acids.

Similarly, in our earlier studies myristic acid seemed much more potent than palmitic acid. In this series, they average out to be about equally effective. In only two tests did myristic appear substantially more hypercholesterolemic than palmitic.

Table 89

Composition of the Diets Studied and the Changes Induced in Serum Cholesterol Levels

Period	Diet	No.	Fatty Acid Content: Saturated Fatty Acids <12:0 (S_{10})	12:0 (S_{12})	14:0 (S_{14})	16:0 (S_{16})	18.0 and over (S_{18})	Total S	Total M	Total P	Dietary Cholesterol	Serum Cholesterol Δ
	Group I		% of Total Calories								Mg/Day	Mg %
1	Olive	10	0.00	0.05	0.10	5.46	1.56	7.17	26.78	4.25	306	−9.5
2	Olive + 16	10	0.00	0.05	0.10	12.43	1.43	14.01	20.22	3.71	306	+17.0
3	Olive + 12	10	0.00	8.70	0.26	4.62	1.53	15.11	19.46	3.64	306	+9.8
4	Safflower	10	0.00	0.01	0.13	3.74	1.40	5.28	6.97	25.93	306	−43.3
5	Safflower + 16	10	0.00	0.04	0.17	11.51	1.17	12.86	6.21	19.09	306	−21.2
6	Safflower + 12	10	0.00	8.37	0.20	3.12	1.10	12.79	5.89	19.51	306	−28.5
7	MCT	10	31.89	0.13	0.30	1.46	0.58	34.16	2.77	1.23	306	−3.2
8	Butterfat + 18	10	0.96	0.61	2.70	10.80	9.79	25.06	10.18	2.60	442	+31.1
9	MCT + 14	10	18.47	0.08	13.76	1.49	0.55	34.35	2.70	1.10	306	+32.6
10	Saf-MCT + 14 50-50	9	9.21	0.04	6.95	2.64	0.97	19.81	4.84	15.52	306	−13.6
11	Olive-MCT + 14 50-50	9	9.21	0.04	6.93	3.48	1.06	20.73	14.75	2.68	306	+13.2
12	Saf-MCT + 18 20-80	9	13.89	0.30	0.24	3.08	10.25	27.76	4.29	6.02	306	+14.9
13	Olive-MCT + 18 20-80	9	13.89	0.30	0.23	3.43	10.29	28.14	8.21	1.68	306	+19.7
14	MCT + 16	9	17.89	0.08	0.33	14.76	0.74	33.80	3.19	1.10	306	+42.8
15	Olive-MCT + 16 20-80	9	14.08	0.30	0.28	12.91	0.91	28.48	7.83	1.79	306	+21.9
	Group II											
16	Olive	11	0.06	0.05	0.10	5.46	1.56	7.17	26.78	4.25	306	−12.9
17	Olive + 18	11	0.00	0.05	0.10	5.20	8.25	13.58	21.06	3.57	306	+12.1
18	Olive + 14	11	0.00	0.08	8.04	4.91	1.49	14.52	20.04	3.64	306	+15.2
19	Safflower	11	0.00	0.01	0.13	3.74	1.40	5.28	6.97	25.93	306	−41.9
20	Safflower + 18	11	0.00	0.04	0.13	4.00	8.16	12.33	6.55	19.22	306	−19.8
21	Safflower + 14	11	0.00	0.14	7.84	3.38	1.20	12.56	5.98	19.59	306	−19.0
22	MCT	9	31.89	0.13	0.10	1.46	0.58	34.16	2.77	1.23	306	−11.9
23	Butterfat	9	2.60	1.31	4.00	10.83	4.78	23.52	12.69	1.95	332	+33.3
24	MCT + 18	9	17.34	0.40	0.26	2.92	12.46	33.38	3.61	1.04	306	+5.1
25	Saf-MCT + 18 50-50	9	8.60	0.20	0.20	5.29	6.93	19.22	5.29	13.49	306	−12.6
26	Olive-MCT + 18 50-50	9	8.68	0.20	0.18	4.19	7.01	20.26	15.21	2.64	306	+9.6
27	Saf-MCT + 14 20-80	9	14.79	0.06	11.04	1.94	0.72	28.55	3.56	6.02	306	+31.6
28	Olive-MCT + 14 20-80	9	14.79	0.06	11.05	2.29	0.76	28.93	7.53	1.74	306	+31.2
29	MCT + 16	9	17.89	0.08	0.33	14.76	0.74	33.80	3.19	1.10	306	+39.1
30	Saf-MCT + 16 20-80	9	14.08	0.30	0.29	12.56	0.88	28.11	3.87	6.12	306	+21.6

We should like to emphasize again, as we did in the earlier paper, that even under these conditions where the change in serum cholesterol is a mean value of that obtained with 9 to 11 men, these values have a standard deviation of about 5 mg %. Thus, if the confidence limits are about ± 2 standard deviations, we might occasionally miss the true value by about 20 mg %. There are substantial differences in the way men respond and in the level of serum cholesterol in a man from day to day in addition to the technical errors of measurement of serum cholesterol. It is clear that comparisons based upon any 2 or 3 periods may have relatively little quantitative meaning. Much of the literature is based upon such comparisons. It seems to us quite clear that one cannot generalize from either a few individuals followed through a series of tests or groups tested with limited numbers of oils.

The data from this series of oils have been analyzed by multiple regression analysis. Table 90 shows the regression coefficients for a variety of equations including various fatty acids and the degree to which the equation explains the variance in the data. The overall data including 30 test periods has a total variance of 16.647 to be explained. The residual sum of squares, the variance not explained by the equation, is shown in column 9 of Table 90. R^2 (column 10) is the percentage of variance explained by the equation, and R, the multiple coefficient of correlation is shown in column 11.

In this series, the polyunsaturated fatty acids (almost entirely linoleic acid) was the most important variable. Thus, the equation, line 1 of Table 90

$$\text{Cholesterol} = -2.45\,P + 2.60$$

explains approximately 67% of the total variance found. As additional variables are added, the equations become more efficient predictors of the changes found. The addition of palmitic acid (S_{16}) alone to the equation (line 2) improves the predictability substantially as does the addition of myristic acid (S_{14}) (line 3). A substantial improvement occurs when both of these saturated fatty acids and polyunsaturated fatty acids are included. The further addition of stearic acid (S_{18}) results in further improvement and this equation (line 5) explains slightly over 90% of the total variance in the data. The data (or the analysis we have applied) do not permit a clear decision to be made on whether any of the other variables, lauric acid (S_{12}), the monounsaturated acids (M), or the shorter chain saturated acids (S_{10}), have any significant effect upon the serum cholesterol level.

DISCUSSION

Considering these data in relation to previous publications, especially those of Keys *et al.* (1965A, 1965B, 1965C, 1965D) and of Hegsted *et al.*

Table 90

Characteristics of Regression Equations Including Different Variables

Column →	1	2	3	4	5	6	7	8	9	10	11
	<12:0 (S_{10})	M	12:0 (S_{12})	18.0 and over (S_{18})	14:0 (S_{14})	16:0 (S_{16})	P	C (Constant)	Residual Sum of Squares	R^2*	R
1							−2.45	2.30	5532	0.6671	0.817
2						1.73	−2.27	3.06	3093	0.7601	0.872
3					1.20		−2.38	0.87	4852	0.7085	0.842
4					2.14	2.47	−2.06	0.18	2031	0.8742	0.935
5				1.19	2.61	2.81	−1.92	3.61	1632	0.9020	0.950
6			0.46		2.19	2.51	2.06	−0.11	2258	0.8768	0.936
7		−0.46			1.99	2.44	−2.16	−2.64	1798	0.8926	0.945
8	0.12				2.15	2.54	−1.97	−0.78	2067	0.8768	0.936
9			0.87	1.30	2.25	2.90	−1.92	3.35	1539	0.9076	0.953
10	0.22			1.38	2.74	3.02	−1.70	1.88	1492	0.9104	0.954
11		−0.48	0.64		2.05	2.48	−2.17	−3.18	1757	0.8957	0.946
12		−0.43	1.01	1.22	2.59	2.85	−2.05	0.39	1276	0.9233	0.961

* Proportion of the variance explained by the equation.

(1957) leads to several important conclusions. In the first place, the ability of changes in any single fatty acid to predict changes in the level of serum cholesterol in any limited series of oils is largely happenstantial. Thus, in our earlier publication, myristic acid alone was found to be the single variable which explained most of the variance whereas in this series the changes in polyunsaturated acids is the most important. Clearly these differences depend then upon the composition of the particular oils that one happens to test. The fewer the oils studied, the more restricted the composition of the oils studied, the more likely it is that one will arrive at untenable conclusions. Presumably the reason myristic acid stood out in our previous series was that coconut oil was the saturated oil used in most of the tests. The emphasis Kinsell *et al.* (1958) place upon the polyunsaturated acids also reflects the oils they have studied.

Secondly, the conclusion of Keys *et al.* (1965A) that stearic acid has no effect upon the level of serum cholesterol would appear to rest upon too few tests. The fact that we reached a similar conclusion also reflects the fact that there were not enough oils in the series in which stearic acid was varied. We must conclude from the data presented in this paper that stearic acid does have an effect. The inclusion of stearic acid in the predictive equations always improved the fit with the data. Stearic acid appears to have approximately half the effect of an equal amount of palmitic or myristic acid.

Thirdly, it should be noted that the role of lauric acid is not well defined in this series because relatively few oils containing it were studied. Based upon the results of periods 5 and 6, lauric acid appears to have a hypercholesterolemic action (Table 89).

Finally, we would emphasize again that although one can certainly identify the primary variables of importance, it is extremely difficult to study the variables of less importance and one should continue to view the regression coefficients with skepticism. The major difficulties are perhaps three in number:

(1) It may be impossible to design a perfectly balanced study in which the large number of fatty acids are changed so that there are no significant correlations between the independent variables. The series reported here is the most extensive attempt so done but we have not succeeded entirely. As long as there is this [illegible] constraint in the data, we [illegible] be certain [illegible] what the regression coefficients mean.

(2) In multiple regression analysis [illegible] independent [illegible]

[illegible] recognized.

[illegible] cholesterol [illegible] in [illegible] the diet [illegible] and this [illegible] Our data are [illegible] 9 to 11 [illegible] the diets [illegible] 27, and 29 were [illegible] predicted and indeed [illegible] predict these points [illegible] These [illegible] that these points are [illegible] 22 and 27 [illegible] 14 and 29 are duplicate [illegible] with the two groups of [illegible] diets suggests the same conclusion. One can, of course, measure the variance within groups but we are now talking about the difference between the serum cholesterol measured and the level of serum cholesterol that ought to

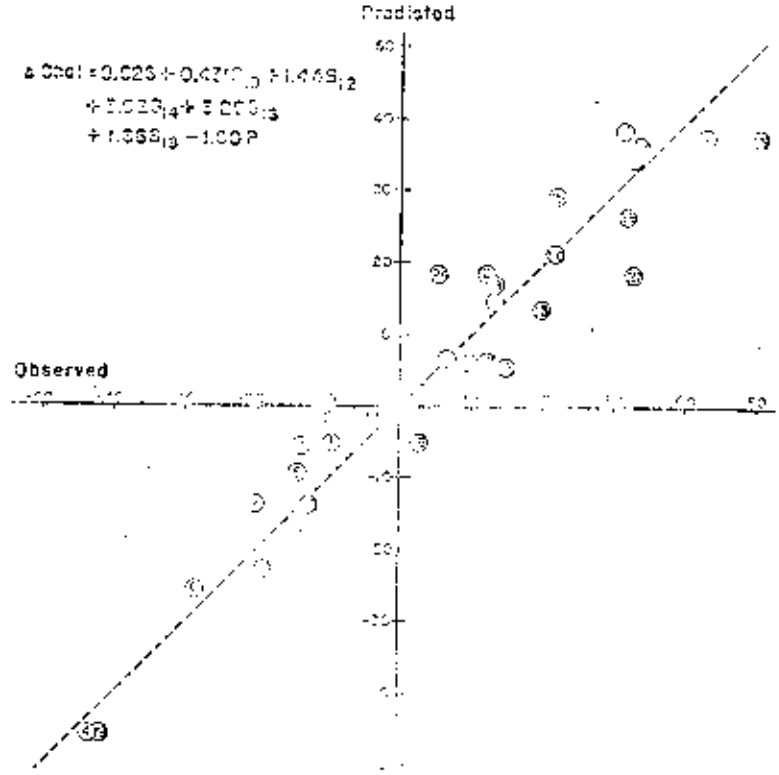


Fig. 55 Predicted [illegible]

be found. This can only be determined by repeated duplication with groups of men. Such data are not available in sufficient numbers in any studies with man so far reported.

CONCLUSIONS

The effects on the level of serum cholesterol of a series of semisynthetic oils are reported. These oils have been deliberately chosen to yield large variations in the content of the saturated fatty acids, variations which cannot be found in natural fats. Distinctions between the effects of specific fatty acids on serum cholesterol should be possible with such materials. Contrary to previous studies by ourselves and others, in this series of oils stearic acid was a significant factor in elevating the serum cholesterol and myristic acid appeared to have approximately the same effect as palmitic acid. The effects of lauric acid and shorter chain saturated fatty acids, or of the monounsaturated fatty acids, if any, were not clearly definable.

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Foreign-Body Type Reaction in Fat Cells.* (24039)

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During studies of the response of rats to diets containing very high amounts of saturated fats, a foreign-body type reaction was observed in fat cells of adipose tissue adjacent to sections of various organs prepared for histological examination. This report describes these altered fat cells and various dietary conditions under which they occur. Although a similar abnormality had been observed earlier with one of these fats by Cox and DeEds,† their data have not been published.

Methods and materials. Weanling male and female rats were obtained from Holtzman Rat Co. and fed one of the following diets. Each diet contained 20% vitamin-test casein, 4% B-vitaminized casein(1), 5% USP XIV salts, 4% corn oil, 17% glucose, and supplements of Vit. A, D, and E at levels of 40,000 I.U., 4,000 I.U., and 510 I.U./kg diet, respectively. The saturated fats incorporated at a level of 50% were fully-hydrogenated lard, palmitic acid, stearic acid, or a saturated partially acetylated monoglyceride(1). Ethyl stearate was fed at 45% with 5% cellulose added to provide body to the diet. A control group was given 50% lard. Rats were sacrificed at 8-week intervals and examined grossly. Portions of perigastric, perirenal, periadrenal, perigonadal, and subcutaneous fat were removed from each rat, fixed with Bouin's solution, sectioned, and stained with hematoxylin and eosin.

Results. Gross examination of the viscera and body cavities was not remarkable except for general fullness of the gastrointestinal tract with creamy colored food and an occasional mottled appearance of fat depots in animals fed the saturated fats.

Microscopic examination of sections showed an unusual foreign-body type reaction in fat from rats fed saturated fats (Fig. 1, top). The lesion consisted of a multinucleated cellular reaction usually located within the fat cell membrane with nuclei arranged evenly about periphery of the fat cell or in clumps. In many instances, the material which occupied the center of the fat cell, appeared to have been partially dissolved, leaving delicate slit-like spaces which were radially oriented and suggestive of fatty acid crystals (Fig. 1, bottom). The reactive process usually involved 2 or more adjacent fat cells and was separated from similar areas by uninvolved fat cells. No acute inflammatory reaction, evidence of recent hemorrhage, or birefringent material were associated with the fat reaction.

The phenomenon occurred within 8 weeks in rats fed diets containing palmitic acid, stearic acid, ethyl stearate, or saturated partially acetylated monoglycerides and within 16 weeks in rats fed the fully-hydrogenated lard diet, but was not observed in rats fed the control lard diet. The flecks of opaque white material observed grossly in the depot fat of some rats were associated with the severest occurrence of the foreign-body type reaction as seen microscopically.

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* Communication No. 247 from Research Labs., Distillation Products Industries.

† Observed in rats fed saturated acetylated monoglycerides for long periods of time by A. J. Cox, Dept. of Pathology, Stanford Univ., Calif., and F. DeEds, Western Regional Research Laboratory, Albany, Calif., private communication. *Added in proof:* Recently published by Cox and DeEds, *Am. J. Path.*, 1958, v34, 263.

FIG. 1. *Top:* Perirenal fat showing multiple islands of fat injury separated by normal fat. Hematoxylin and eosin, ×60. *Bottom:* Higher magnification showing variations of cellular reaction. Multinucleated giant cells are present at top of print. A fat cell containing radial slit-like spaces is seen near bottom of print. Hematoxylin and eosin, ×430.

This reaction in visceral and retroperitoneal fat of the rat bears some histologic resemblance to pathologic conditions of human fat such as fat necrosis of newborn(2), sclerema neonatorum(3), and traumatic fat necrosis of various types(4,5,6). Further studies are underway to determine the histochemical characteristics of the lesion.

Appearance of the foreign-body type reaction in all groups except that fed lard indicates that the reaction is a response to a relative or absolute excess of saturated long chain fatty acids. We are testing diets containing various proportions of saturated to unsaturated fatty acid to determine the critical ratio which produces this reaction.

Summary. A foreign-body type reaction has been observed in fat from rats fed diets containing high levels of saturated fat. The phenomenon and the dietary conditions under which it was produced, are described.

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Effect of Dilantin Sodium on Tensile Strength of Healing Wounds.* (24046)

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Hyperplasia of the oral gingiva is a common reaction among epileptics following systemic administration of sodium diphenylhydantoinate (Dilantin Sodium). The phenomenon does not occur in all patients and, in those affected, there is a wide range in severity of the lesion(1,2). The reason for this selective involvement of gingival tissues, other tissues throughout the body apparently being unaffected, is unknown nor is the mechanism of hyperplasia understood. Tissue reaction is basically a fibrous hyperplasia with formation of increased bulk of dense collagen fibers. This has suggested to Shapiro that the compound might favorably influence the healing of gingival wounds through enhancing collagenization(3). He reported that administration of dilantin to non-epileptic patients in-

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CARCINOGENESIS BY CHOLESTEROL

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THE existence of carcinogens of biological origin was demonstrated for the first time by Shabad (1937) who induced sarcoma in mice by injecting lipoid extracts from post-mortem human tissues. His discovery was confirmed by a number of other investigators (des Ligneris, 1940; Hieger, 1940; Steiner, 1941; Sannié, Truhaut, Guérin and Guérin, 1941) who found that sarcomas in mice resulted from the subcutaneous injection of cholesterol-rich extracts, e.g. (1) the unsaponifiable part of the fatty fraction of liver, kidney, muscle or lung of human subjects who had died of cancer or of some other cause, or (2) the unsaponifiable fraction from the brain and spinal cord of cattle. The succeeding steps in this work showed that cholesterol itself, even when highly purified had carcinogenic activity (Hieger, 1947, 1949, 1957; Hieger and Orr, 1954). In a recent publication, Hieger (1957) describes an experiment in which 11 mice developed sarcoma out of 115 (initially) injected mice: if the incidence is calculated on the number of mice which survived to the minimal latent period (thus cancelling out from the initial number all mice which died early and were not at risk) the incidence amounted to 14 per cent. The cholesterol in this experiment was highly purified (Schwenk process) and administered as a 10 per cent solution in olive oil prepared by heating on the water bath. In control experiments where the solvent alone was injected into mice, (lard was used as solvent in the earlier tests and controls), 5 sarcomas developed in a total of 1122 treated mice (Table I).

TABLE I.—*Control Tests on Solvents Injected Subcutaneously into Mice*

Solvent	Strain of mice	Number at start	Survivors (months) 12	18	24	Sarcomas
Lard	stock / C_{57}	366	169	81	16	1
,,	stock	100	47	20	2	0
Olive oil	C_{51}	134	90	43	6	1
,,	stock	50	33	12	2	0
,,	,,	50	24	13	0	0
,,	,,	66	14	1	0	0
,,	,,	56	47	20	1	3
,,	C_{57}	50	39	28	13	—
,,	C_{57} / stock	58	52	33	7	0
Tricaprylin	C_{57}	30	17	6	2	1
Stearic acid + olive oil	stock	51	32	13	2	0
Tristearin + olive oil (5 : 30)	,,	50	33	13	3	1
Sesame oil	,,	81	46	13	4	0
Total		1122				5

The reports of the carcinogenic activity of cholesterol have provoked comment and criticism. For example, (I) it has been objected that since cholesterol is present in all tissues of the body and particularly in atheromatous arteries, cancer should be expected to occur in all tissues and particularly at atheromatous sites. (II) A second criticism takes another form; since cholesterol is found in all tissues but cancer does not appear in every tissue, the carcinogenic activity of cholesterol when injected into mice could be due to an active derivative formed from the cholesterol by some reactions peculiar to the conditions of the experiment. (III) A third objection suggests that as the threshold carcinogenic dose of benzpyrene for the mouse is of the order of only a microgram the activity of cholesterol in the mouse experiments could be due to contamination with a minute trace of a potent carcinogen of the type of benzpyrene. (IV) A fourth comment points out the difficulty of reconciling the negative results obtained by other workers who tested cholesterol by more or less the same technique which gave positive results in Hieger's experiments. (V) Fifthly, the idea has been put forward that since watery suspensions of cholesterol have proved negative when tested for carcinogenic activity and since in tests giving positive results the cholesterol is heated with oily solvent at water bath temperature for up to 2 hours before the solution is injected, chemical changes could well take place during the heating which might at least contribute to the carcinogenic activity of the solution.

These five questions will now be discussed.

(I) *Cholesterol present in all tissues of the body*

Cholesterol is found associated with fat in all tissues and particularly in atheromatous aortas, yet cancer in man develops at sites of which the aorta is one of the least common. The apparent contradiction between this fact and the positive mouse tests on cholesterol is by no means insoluble. Urethane when given to mice in the drinking water or painted on the skin, gives rise to tumours of the lung only; β-naphthylamine when inhaled or absorbed through the skin by men working in the dye and rubber industries causes cancer of the bladder and not of other organs; benzpyrene applied in large doses over a space of several years failed to induce cancer in the skin of the monkey. When discussing carcinogenic action, the tissue, and the species of animal to which it belongs as well as the type of carcinogen must all be taken into consideration. The human aorta contains very little cellular tissue which may well be resistant to carcinogenesis by cholesterol. The inferences which can be made from the work on cholesterol are not intended to suggest that all human cancer is mediated by cholesterol, no such crude hypothesis is propounded here, the illustrations just quoted merely show that the *opposite* conclusion has not been established.

(II) *Injected cholesterol as the precursor of a carcinogen*

In its simplest form, question (II) suggests that although cholesterol itself is a normal chemical component of the tissues, when it is injected subcutaneously in oily solution it is slowly converted to some active derivative which is the true carcinogen.

This possibility gains support from Bischoff's experiments on the reported carcinogenic capacity of oxidative derivatives of cholesterol which he obtained as a by-product in the preparation of progesterone by the oxidation of cholesterol

with permanganate. The mixture of compounds resulting from the reaction induced sarcomas on injection subcutaneously into mice of the Buffalo strain. From this result Fieser *et al.* (1955) concluded that the active agent in carcinogenesis by cholesterol was some ketonic derivative (e.g. Δ^4-cholestene-3-one) or a chemically active isomer, namely, lathosterol (Δ^7-cholestenol). Fieser *et al.* (1955), Bischoff and Rupp (1946), Bischoff, Lopez and Rupp (1954), and Bischoff *et al.* (1955) reported that a number of derivatives of cholesterol when injected in batches of 30 Buffalo mice induced 19–60 per cent of fibrosarcomas. The most potent member of this group of compounds was 6β-hydroperoxy-Δ^4-cholestene-3-one. They state that purified cholesterol was found inactive but that unpurified cholesterol had some carcinogenic potency.

> "Administered in sesame oil, impure cholesterol lathosterol, and the non-keto fraction of cholesterol oxidized by oxygen in soap suspension produced higher incidences of fibrosarcoma than controls which received only sesame oil or sesame oil plus pure cholesterol or cholestenone."

The results of Bischoff and his colleagues are summarised in Table II.

TABLE II.—*Oxidative Derivates of Cholesterol as Carcinogenic agents (Fieser. et al., 1955 ; Bischoff, et. al., 1955)*

Compound tested	Per cent fibrosarcomas (in groups of 33 Buffalo mice)
none, control	0
6-hydroxy-Δ^4-cholestene-3-one	19
Δ^4-cholestene-3,6-dione	34
none, control	0
cholesteryl oxide	43
6-hydroperoxy-Δ^4-cholestene-3-one	60

Some of the compounds reported to be strongly carcinogenic by Fieser and Bischoff have been tested in the writer's laboratory. The yield of tumours does not confirm the statement of the American workers that these oxidative derivatives are more potent carcinogens than cholesterol, neither can we agree with their pronouncement on the lack of potency of cholesterol. It must be noted however that different strains of mice were used in the two laboratories.

In an earlier paper (Hieger, 1959) giving the results of experiments in progress it was stated that none of the compounds tested had at that stage produced sarcomas ; the experiments are now almost completed and a number of tumours have developed, 5 of them in the series of mice injected with hydroperoxide.

The results are shown in Table III ; they indicate that (1) under our conditions only the hydroperoxide of all the oxidative derivatives of cholesterol has given evidence of any appreciable carcinogenic activity ; (2) mice of C_{57} strain are more sensitive than stock mice, but this difference probably depends to some extent on their longer life span. The same difference of response between C_{57} and stock mice was observed when two parallel series were tested with cholesterol purified by the Schwenk process (Table IV). Although C_{57} mice were used for the tests on Δ^5 : cholestene-3-one and lathosterol, the yield of tumours totalled a single sarcoma ; the crude $KMnO_4$ oxidation product, Δ^4-cholestene-3 : 6-dione and 6-hydroxy-Δ^4-cholestene-3-one were not tested on C_{57} mice and it is possible that they too might have given some positive results under such conditions.

TABLE III.—*Sarcoma Induction by Derivatives of Cholesterol*

Compound	Number of mice and strain		Survivors (months) 12	16	18	21	24	30	Sarcomas
Crude $KMnO_4$ oxidation product of cholesterol	stock	50	24	17	14	3	1	0	0
Δ^4-cholestene-3 : 6-dione (in olive oil)	,,	55	31	22	16	8	4	0	0
6β-hydroperoxy-Δ^4-cholestene-3-one (in olive oil)	,,	55	28	22	18	11	5	0	0
(In sesame oil)	,,	65	32	27	22	14	8	—	0
,,	,,	51	34	31	15	8	2	—	0
,,	Buffalo	36	7	4	2	1	0	—	1
,,	C_{57}	50	46	39	38	23	15	—	4
,,	stock	50	35	28	13	9	0	—	1
6-hydroxy-Δ^4-cholestene-3-one (in olive oil)	,,	54	30	23	21	4	0	—	1
(in sesame oil)	,,	40	14	12	8	4	0	—	0
Δ^5-cholestene-3-one (in olive oil)	C_{57}	50	38	34	29	23	11	—	1
,,	C_{57}	50	39	—	17	—	5	—	0
Lathosterol									

TABLE IV.—*Sarcoma Induction by Purified Cholesterol (Schwenk Process) Injection in C_{57} Mice Compared with Negative Results in Stock Mice.*

No. of mice	Strain	Survivors (months) 12	18	24	30	Sarcomas
57	Stock	25	14	6	1	0
50	C_{57}	47	31	24	7	3 (latent periods 12, 18, 24 months)

As far as the results go they suggest that the hydroperoxide and pure cholesterol have about the same order of carcinogenic activity (Tables III and IV) which gives no support to the idea expressed by Fieser *et al.* (1955), and is implicit in the writings of Bischoff *et al.* (1955), that cholesterol is a pre-carcinogen when injected into the mouse and has to be converted to an oxidised carcinogenic derivative before it can induce sarcoma.

(III) *Trace contamination by carcinogens of hydrocarbon type*

The experiments designed to test lipoid tissue-extracts and the earlier tests with cholesterol before 1950 were carried out in a room at the Chester Beatty Institute where some other series of mice were being treated with potent carcinogens such as benzpyrene, thus introducing the possibility of contamination. In 1950 all the experiments on carcinogenesis by lipoids and steroids were transferred to our country laboratories, twenty miles out of London, where the atmosphere is less smoky and where there are arrangements for housing the mice in rooms uncontaminated by carcinogens other than cholesterol (Hieger and Orr, 1954). Nevertheless, it soon became clear that even if all reasonable precautions were taken to avoid contamination, cholesterol still induced sarcoma in mice. In order to obtain some data on the degree of contamination which might appreciably affect the yield of sarcomas with cholesterol alone, series of mice were given small doses of benzpyrene to find where the threshold dose of benzpyrene lay (Table V).

The results of the experiments detailed in Table V suggest that (1) the threshold dose of benzpyrene is of the order of 1 γ for C_{57} mice or stock mice, (2) an amount

of benzpyrene in the region of 10 γ would have to be injected along with the cholesterol in order to give a yield of sarcomas appreciably higher than what would be expected with cholesterol alone, (3) the sensitivity of mice to small doses of benzpyrene can vary by a factor of at least 5 to 1.

TABLE V.—*Threshold Dose of Benzpyrene Required for Sarcoma Induction*

Experiment	No. of mice at start	Strain	Total dose of benzpyrene (in 10% cholesterol : 90% olive oil)	How administered	No. of sarcomas
1	50	C_{57}	0·04 γ	in a single dose	0
2	"	"	0·4 γ	"	1
3	"	"	4·0 γ	"	5
4	"	"	40·0 γ	"	23
5	"	stock	7·5 γ	divided in 6 injections	2
6	"	"	0·75 γ	"	3
7	"	"	0·075 γ	"	3
8	"	"	50 γ	"	19
9	"	"	5 γ	"	3
10	"	"	0·5 γ	"	0
11	"	"	0	"	0
12	"	"	0	"	0
13	"	"	10 γ	"	1
14	"	C_{57} stock	10 γ	"	10
15	"	"	10 γ	in single dose	6

If it be assumed as a working hypothesis that sensitivity is proportional to the yield of tumours and inversely as the dose of carcinogen required, experiments 3, 13, 14 and 15 (Table V) suggest that C_{57} mice are of the order of 10 times as sensitive as stock mice to threshold doses of benzpyrene when it is administered in 5 divided doses, but only about twice as sensitive when the dose is given in one injection; experiments 9 and 13 suggest that the sensitivity of different lots of stock mice can vary by something of the order of 6 : 1 or more. Although the benzpyrene dose varied in the ratio 1 : 100 in experiments 5, 6, 7, the yield of tumours was much the same, suggesting that the cholesterol in the injection material was the active carcinogen and not the benzpyrene. In these three experiments the incidence was about 6 per cent calculated on the number of mice at start, but about double this figure when the calculation is based on the mice which survived a minimum of 1 year and therefore were effective. (i.e. at risk). An incidence of 10–15 per cent would agree well with that obtaining in several of our experiments where the mice developed a reasonable proportion of tumours when injected with cholesterol (the frequently occurring incidence of about 10–15 per cent will be further discussed in a forthcoming publication). This figure of 10–15 per cent can now be contrasted with the results for experiments 10, 11, 12 where the incidence was 0 per cent. As far as the data go, they suggest that the sensitivity of different batches of mice can vary over a considerable range whether this sensitivity is assayed by the use of cholesterol or by small doses of benzpyrene.

To extend the idea of possible contamination by benzpyrene a further step, the following experiment was carried out: mice were injected with cholesterol and arranged in groups of 4 in separate cages; to each cage was then added a single mouse of a different colour (a coloured mouse with 4 white mice or a white mouse with 4 coloured mice) which was painted biweekly with benzpyrene solution in the interscapular area during the whole course of the experiment. The

mice injected with cholesterol were thus in an environment contaminated with benzpyrene, yet only in experiment A (Table VI) was there a large number of sarcomas which would be attributed to benzpyrene. In this particular experiment, the 11 injections of cholesterol were given at 4 weekly intervals concurrently with the biweekly painting of the single mouse with benzyprene. A possible explanation of the results in experiment A is that at the injection of cholesterol some benzpyrene on the fur, transferred there by contact with the painted mouse, was pushed through the skin into the subcutaneous layers. In a few cases papillomas arose on the skin of the mice which had not been painted with benzpyrene but had a cage-mate which was so treated.

TABLE VI.—*Effect of Exposing Mice Injected with Cholesterol to an Environment Contaminated with Benzpyrene*

In each experiment below the mice were caged in groups of 5. Four mice were for injection *only*, and the fifth for painting *only* (with benzpyrene).

	Strain	Survivors: Start	12 (months)	18	24	Sarcomas
A Injected 11 times with 10 per cent cholesterol in olive oil at beginning of each of 11 months. In the 2nd and 3rd week of each month—biweekly painting	stock	80	42	16	—	12
B 4 injections (cholesterol) at fortnightly intervals, then after a 3-week interval painting begun	C_{57}	40	30	26	3	0
C 4 injections (cholesterol) at fortnightly intervals, then after a 3-week interval—painting begun.	stock	40	30	13	2	2
D 4 injections (cholesterol) at fortnightly intervals, then after 4 months—painting begun.	stock	40	28	12	2	0
E Paint biweekly for 3 months only. Then after an interval of 1 month, 4 fortnightly injections of olive oil—tristearin (5 : 1).	stock	40	12	2	—	0
F Inject 4 times with an olive oil : tristearin mixture (5 : 1), then after an interval of 3 weeks, biweekly painting begun.	stock	44	28	14	2	2
G Painted biweekly for 3 months. Then, after an interval of 2 weeks given 4 injections of cholesterol.	stock	60	11	—	—	0

EXPLANATION OF PLATES

FIG. 1.—Sarcoma induced at site of injection of purified cholesterol in olive oil. ♀ stock mouse. 16th month. × 65.

FIG. 2.—Same tumour as in Fig. 1. × 300.

FIG. 3.—Sarcoma induced at site of injection of purified cholesterol in olive oil. ♂ stock mouse. 18th month. × 65.

FIG. 4.—Same tumour as Fig. 3. × 300.

FIG. 5.—Sarcoma induced at site of injection of purified cholesterol in olive oil. ♂ stock mouse. 9th month. × 75.

FIG. 6.—Same tumour as in Fig. 5. × 300.

FIG. 7.—Tumour on *left* side of ♀ stock mouse which had been injected on *right* side with tristearin in olive oil. 19th month. × 90.

FIG. 8.—Same tumour as in Fig. 6. × 300.

FIG. 9.—Tumour on *left* side of ♀ stock mouse which had been injected on *right* side with cholesterol in olive oil containing added 1 per cent turpentine. 19th month. × 90.

FIG. 10.—Same tumour as in Fig. 9. × 300.

FIG. 11.—Tumour on *left* side of ♂ stock mouse which had been injected on *right* side with cholesterol in olive oil. 15th month. × 90.

FIG. 12.—Same tumour as in Fig. 11. × 300.

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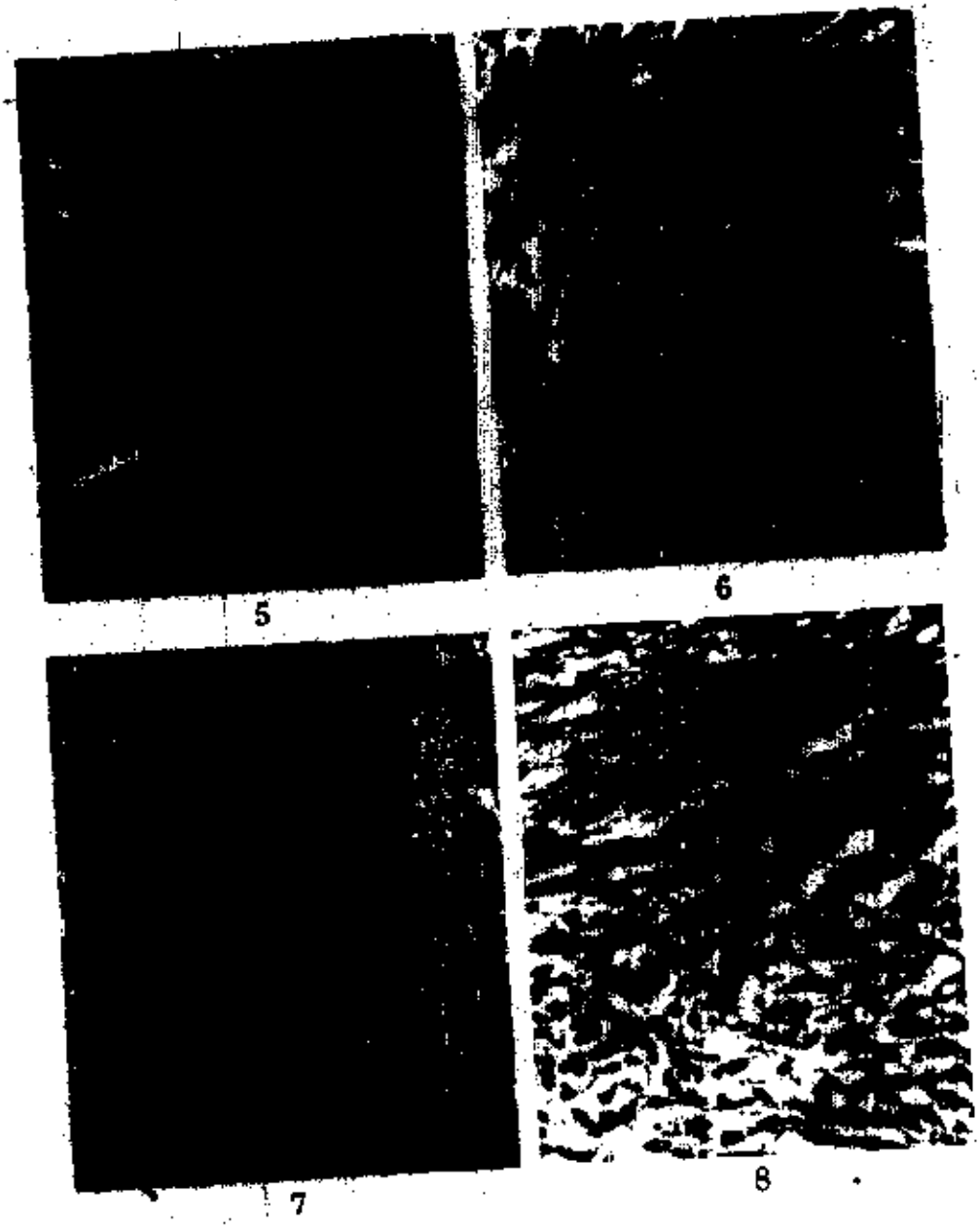

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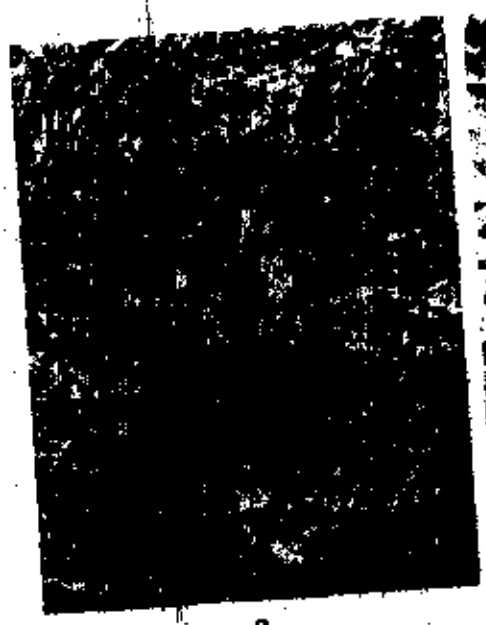

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These data show that no very stringent precautions need to be taken to avoid fictitious results due to contamination, since the mice in experiments B and C, although in an environment contaminated with benzpyrene, did not develop sarcomas in excess of what would be expected from the injection of cholesterol *per se*. It is difficult to explain the appearance of the two sarcomas in experiment F (Table VI) where no cholesterol but a mixture of two fats were used for injection (olive oil and tristearin) in order to test the possible localising effect of lipoid substances other than cholesterol.

(IV) *In other laboratories, cholesterol has not produced tumours*

Hartwell (1951) quotes 39 entries under the section dealing with tests for the carcinogenicity of cholesterol; some details are given of the species of experimental animal, vehicle and dose of cholesterol, site and route of administration, duration of experiment, survival rate and yield of tumours. None of these 39 experiments gave positive results of the kind which are the criteria of the present enquiry, i.e. spindle-celled sarcoma at the site of injection (sub-cutaneous) in mice injected with large doses of cholesterol of the order of a minimum of 40 mg. administered in lard or olive oil, in mice of stock or C_{57} strain which had a satisfactory survival rate. However, Hartwell's compilation was not completely up to date even in 1951 since he does not refer to the writer's experiments published in 1947 where 11 sarcomas were obtained by the injection of commercial cholesterol in 144 mice (Hieger, 1947).

As far as the author is aware, his experiments are almost the only ones giving positive results in tests for the carcinogenic activity of cholesterol. Is the discrepancy due to differences in the solvent, in the strain of mouse, in the duration of the experiment or in the environmental conditions of the animals? The role played by the solvent in carcinogenesis by lipoid substances and steriods has not yet been settled. Cholesterol of different degrees of purity has been found highly active when administered in olive oil or in lard; Steiner (1941) reported that mixtures of the unsaponifiable fractions of human liver with tricaprylin or with sesame oil were strongly carcinogenic; Bischoff *et al.* (1955) tested oxidative derivatives of cholesterol suspended in sesame oil with positive results. These four oils (lard, olive oil, sesame oil and tricaprylin) can accordingly be considered as suitable vehicles for testing carcinogens of lipoid or steroid type since the control tests on the oils alone give a very low yield of tumours, namely, of the order of ½ per cent (Table I).

The choice of the strain of experimental mouse is probably an important factor in affecting the response to lipoid carcinogens. Cholesterol in olive oil or in lard has given an incidence of 10–15 per cent or more of sarcomas in C_{57} mice, in mixed commercial stock mice, and in the laboratory stock bred from a mixed commercial stock. In an experiment on 100 mice of the CBA strain injected with commercial cholesterol in olive oil, the yield of sarcomas was less, namely 2 per cent (Table VII). Small series of C_3H mice tested with the same solution proved refractory and small series of mice of the MRC strain reacted positively.

Such differences of sensitivity of different strains of mice to cholesterol as a carcinogen are not unexpected since variations of susceptibility to conventional carcinogens (e.g. hydrocarbon type) are well known and an example is shown in Table V. What is more difficult to explain is the frequently occurring fluctuations in response exhibited by different batches of mice of the same genetic origin.

Differences of sensitivity to carcinogens can easily be found when a minimal dosage of carcinogen is used but when an excessive dose of carcinogen is applied (such as, say, a milligram or even a tenth of a milligram of benzpyrene) anything but gross differences of sensitivity will be masked by the excess of carcinogen; therefore, in order to reveal and assess the comparative sensitivity of different groups of mice, the dose of carcinogen should be at threshold, or near threshold, level. If the carcinogen is of an order of potency much below that of the hydrocarbon carcinogens of the type of benzpyrene, it can be expected that even after the application of large doses, only a fraction of the mice would react and the conditions would be well suited for demonstrating variations of susceptibility which are not likely to appear when hydrocarbon carcinogens are employed, unless in minute doses. In Table VII are shown the fluctuations of response when mice of C_{57} or stock strains were tested with pure or with commercial cholesterol. The yield of sarcomas varied from 0–14 per cent or more in different batches of mice. Experiments t and q (both groups injected with commercial cholesterol) are of special interest: the origin of the mice was the same (a more complete correspondence would have been achieved if the individual members of the two groups had been thoroughly "shuffled"). Group q was housed in a room kept at about 70° F. and water was supplied in bulbs in the usual way; group t was housed in cages well supplied with wood wool, in a room facing north where the temperature followed the external temperature but was not allowed to go below 40° F. and the only water available to the mice was supplied in cabbage leaf. In experiment q the yield of sarcomas was zero in 50 mice, in t it was 5 in 46 mice (initially) amounting to an incidence of 16 per cent calculated on the survivors at one year.

These experiments suggest that environmental factors were of importance in influencing the response to the carcinogen. A comparison of experiments s (pure cholesterol) and q (commercial cholesterol) suggests that purified cholesterol (Schwenk process, see Hieger (1957) for details) is more potent carcinogenically than commercial cholesterol; but it must be noted that in experiment r no sarcomas were induced in 54 stock mice with the olive oil preparation of pure cholesterol, whereas in n and o the incidences were 14 per cent in one case and was calculated to have been the same theoretically in the other (Hieger, 1957). It is very difficult to find any explanation of why the same, or very nearly the same, preparation of cholesterol gives 14 per cent of sarcomas in stock mice in one experiment and 0 per cent in another.

The conclusions to be drawn from the data shown in Table VII are that no single variable—nor the purity of the cholesterol, nor chance contamination with atmospheric carcinogens nor strain differences, nor solvent differences—can be implicated as solely responsible for the fluctuations in carcinogen response; the alternative explanation is that a combination of some genetic, congenital or environmental factors (e.g. temperature and drinking water) are the effective agents, but of course such an explanation has still to be proved adequate.

In Section III the range of variations in susceptibility to threshold doses of benzpyrene was observed to be something of the order of 5 : 1, if the sensitiveness be regarded provisionally as proportional to the percentage yield of tumours and inversely as the dose of carcinogen. Fluctuations of susceptibility of this order of magnitude could explain why some batches of mice do not react under the stimulus of cholesterol as carcinogen; as a first approximation it is suggested that such variations are due to the operation of the same factors which cause variations in

the incidence of "spontaneous" neoplasia in mice, although of course, there is no experimental evidence as yet for this idea. It will be recalled that Tannenbaum and Silverstone (1957) showed that mere alterations in the calorific value of the diet of mice could greatly influence the incidence of spontaneous mammary cancer and of tumours induced with carcinogens.

TABLE VII.—*Table Showing Incidence of Sarcomas in Mice After Subcutaneous Injection with Cholesterol*

Experiment	Institute	Room	Strain	Pure or commercial (comm.) Cholesterol	No. of mice and survivors (months) Initial	12	18	24	Sarcomas	% Incidence on initial No.	Calculated on survivors at 1 year %
a	C.B.	—	C_{57}	comm.	108	68	45	10	10	9·3	14
b	,,	—	stock	,,	45	18	5	3	5	11	28
c	,,	—	MRC	,,	20	16	8	2	2	10	12
d	,,	—	C_{57} + stock	pure	34	18	11	2	1	3	6
e	P.W.	non-C	C_{57}	comm.	60	28	14	3	1	1·7	3·6
f	,,	,,	stock	,,	70	38	18	2	3	4·3	7·9
g	,,	,,	,,	,,	65	57	41	11	3	4·6	5·4
h	,,	C	,,	,,	100	75	45	10	8	8	10·7
i	,,	,,	CBA	,,	100	71	45	2	2	2	2·8
j	,,	non-C	C_{57}	pure	172	126	81	20	5	2·9	4
k	,,	,,	,,	comm.	30	10	7	5	1	3·3	10
l	,,	,,	,,	,,	39	24	16	11	3	7·7	13
m	,,	C	,,	,,	75	46	26	3	4	5·5	8·7
n	,,	,,	stock	pure	115	66	22	10	11	9·5	14
o	,,	non-C	,,	,,	109	all † < 1 yr. (epidemic)			3	3	probably much > 3
p	,,	,,	,,	comm.	53	27	11	0	0	0	—
q	,,	,,	C_{57}	,,	50	38	32	23	0	0	—
r	,,	,,	stock	pure	53	25	12	2	0	0	—
s	,,	,,	C_{57}	,,	50	47	35	22	3	6	6·4
t	,,	low temperature	C_{57}	comm.	46	32	23	12	5	11	16
u	,,	,,	stock	,,	40	19	5	0	0	—	—

C.B. = Chester Beatty Research Institute, London. P.W. = Pollards Wood in Buckinghamshire, 20 miles out of London. Non-C = Non-Carcinogen Room. Carcinogens of conventional type (e.g. benzpyrene) were neither used nor kept in this room. C = Carcinogen room. In this room, besides mice injected with cholesterol, were other series undergoing treatment with benzpyrene. Low-temperature room = This room faces north; temperature not below 40°F. but otherwise follows outside temperature. Water is supplied only as present in fresh cabbage leaf.

Dunn, Heston and Deringer (1956) reported that in the female mice in their colony consisting of C_3H strain bearing the mammary milk agent, C_3Hf lacking the agent, $C_{57}BL$ strain and F_1 and backcross hybrids of these strains, a total yield of 106 subcutaneous fibrosarcomas were found in 4049 mice (initially). These workers quote Cloudman (1941) who, referring to a certain colony of mice stated that "sarcomas were among the more common malignant tumours in the subcutaneous regions, but there were no high-tumour stock, and it was unusual to find an incidence of over 15 per cent." Dunn, Heston and Deringer's (1956) results (106 tumours in 4049 female mice) represent a crude incidence of 2½ per cent sarcomas, but as the average latent period when the tumours occurred was at the advanced age of 22–32 months, which was approximately the average longevity of the mice, the true incidence was very probably a multiple of 2½ per cent,

say 5 per cent or more, since a fair proportion were dead before reaching the latent period and could not be considered at risk.

Some impression of the variations of incidence in their experiments is conveyed by the number of sarcomas in consecutive groups of C_3Hf mice (Table VIII).

TABLE VIII.—*Spontaneous Fibrosarcoma in C_3Hf Mice (Dunn, Heston and Deringer, 1956)*

Genetic composition of foster mother	Number of mice	Number of sarcomas
(a) C_3Hf mice foster-nursed on → C_{57} (100 per cent C_{57} chromatin : 0 per cent C_3H chromatin)	81	3
(b) C_3Hf mice foster-nursed on → Cross-bred (50 per cent C_{57} chromatin : 50 per cent C_3H chromatin)	90	9
(c) C_3Hf mice foster-nursed on → Cross-bred (25 per cent C_{57} chromatin : 75 per cent C_3H chromatin)	64	0

It is very difficult to explain how the variations in the genetic composition of the foster-mothers of the mice in experiments b and c was sufficient to change the incidence of spontaneous sarcoma from a maximum of 10 to 0 per cent.

The foregoing considerations suggest that (1) at low intensity stimulus (cholesterol as carcinogen, or threshold doses of benzpyrene or "spontaneous" sarcoma) several factors together or separately can cause wide fluctuations of incidence of sarcoma ; (2) that of these factors, two can be identified namely the "environment" and the genetic composition of the mice ; (3) that alternatively some other factors are operative, the nature of which is as yet unknown.

These 3 possibilities could explain the disagreement between the results of tests on cholesterol and its oxidative derivatives which were used by Bischoff, *et. al.* (1955) and by the writer. Bischoff *et. al.* (1955) and Fieser *et al.* (1955) report that 6β-hydroperoxy-Δ^4-cholestene-3-one is a potent carcinogen, for it gives sarcoma in over 60 per cent of Buffalo mice (Table II). In the tests described in Table III this compound is shown to be as active as cholesterol, i.e. giving a sarcoma incidence of 8–10 per cent but these experiments were carried out on C_{57} mice. A batch of Buffalo mice imported from the U.S. appeared healthy on arrival but have not maintained this condition, they are very prone to mite infestation (however, vigorous treatment with DDT and with benzyl-benzoate seems to be effecting some improvement of the state of the skin). Bischoff and Rupp (1946) find that this strain of mice is liable to spontaneous sarcoma, they state ". . . In previous experiments male mice observed for 20 months or more developed a higher incidence of skin tumours ; namely 10 per cent in 30 control mice, and 23 per cent in 30 males that had received oestrone." Perhaps the Buffalo mice are specially sensitive to some carcinogens since their subcutaneous tissues are prepared, as it were, for neoplasia, assuming that the two processes follow the same path, but of course, their insensitivity to cholesterol as carcinogen would remain unexplained.

The spontaneous subcutaneous sarcoma rate in the mice in the author's laboratory must be very small indeed. An estimate of the incidence could be made by a count of the sarcomas which have appeared on the *left* side of the animals. All the injections of cholesterol, derivatives of cholesterol, lipoid fractions of tissues and controls on solvents, were carried out on the *right* side. To date over 140 sarcomas have been recorded on the right side and 7 subcutaneous tumours

on the left side, but the histological appearance of most of these 7 tumours is rather different from that of the spindle-celled sarcomas induced at the site of injection by cholesterol and by the lipoid tissue-extracts which are very similar histologically to the sarcomas produced by hydrocarbon-type carcinogens. Consequently, it is doubtful if there is any appreciable incidence of spontaneous spindle-celled sarcoma in our stocks. Photomicrographs of these two kinds of subcutaneous tumour are shown in Fig. 1–12.

Guérin (1954) states that in his colony of 6000 mice the most frequently occurring spontaneous tumour was not mammary cancer but connective tissue tumours of the reticuloendothelial system (275 cases equivalent to an incidence of 4 per cent). Thirteen spontaneous sarcomas were found, 12 of which were "fusohistiocytaire"; the average tumour age was about 20 months.

V. *Role of heating as a possible contributory factor in carcinogenic activity of solution of cholesterol.*

Cholesterol as large dense flakes dissolves slowly on heating with olive oil at water bath temperature and when a large series of mice (say 50–100) are to be injected in turn, the time required for total heating may be approximately 2 hours. During that time chemical changes may occur in the solution; indeed the solutions and even oils alone (particularly sesame oil and lard) after being heated on the water bath show a much increased spectroscopic absorption in the ultraviolet region of the spectrum.

Since our experiments with cholesterol administered in aqueous medium (i.e. finely ground cholesterol in suspension in 4 per cent gelatin gel) have so far given negative results, the criticism has been put forward that chemical changes taking place or initiated during the heating of the cholesterol with oil could explain the carcinogenic activity of the solution and the inactivity of the non-oily suspension. However, there is a total lack of experimental evidence for (and some facts can be advanced which do not support) this possibility. The control tests on the oily solvents alone (olive oil and lard) comprised over 1,000 mice which produced 5 sarcomas only. The oils were heated on the water bath before injection but since they were either liquid or melted very quickly, the time of heating was mainly dependent on the number of mice being injected and therefore the total heating could have been less than in the experiments with solutions of cholesterol; but since the total number of injections was greater in the control series because oil alone is more readily dispersed in the tissues, the effective total heating was probably not very different in the two cases.

To pursue the same line of argument further, it would now be necessary to postulate that the presence of cholesterol during the heating is necessary for inducing the hypothetical chemical change. Sarcomas were not obtained in experiments set up to test this possibility (Table IX). Positive results must take precedence over negative results; the latter may be due to an insensitive batch of mice or to environmental conditions which are not conducive to sarcoma production: some solutions of cholesterol heated in the usual way have *not* produced sarcoma in mice, and if for the sake of argument it be assumed that the agent is indeed formed during the heating, its hypothetical presence therefore is not as decisive as e.g. the sensitivity of the mice or the environmental factor. There seems no reason for multiplying the number of unknowns, and the formation of

a carcinogen by heating oil and cholesterol at water bath temperature remains at present unproved, what evidence exists is against such possibility.

TABLE IX.—*Results of Injecting* (1) *Heated Olive Oil*; (2) *Heated Solution of Cholesterol* (10 *per cent*) *in Olive Oil*

Number of Preparation	No. of mice	Strain	Survivors (months) 12	18	24	Sarcomas
(1)	12 38	C_{57} stock } 50	35	17	6	0
(2)	17 33	C_{57} stock } 50	35	15	4	0
(3)	14 36	C_{57} stock } 50	38	16	9	1

(1) Olive oil heated, with access of air, 75 hours on boiling water bath.
(2) 10 per cent cholesterol + 90 per cent olive oil heated with access of air, 75 hours on boiling water bath.
(3) Olive oil heated, with access of air, 76 hours on boiling water bath then 10 per cent cholesterol added without prolonged further heating.

SUMMARY AND CONCLUSIONS

1. Over 140 sarcomas in mice have been induced by the subcutaneous injection of oily solutions of (1) the unsaponifiable fraction of human tissue, or (2) of cholesterol.

2. Purified cholesterol is at least as potent as the commercial product before purification.

3. In one experiment on highly purified cholesterol, 11 sarcomas developed in 115 mice of which 66 lived over 1 year. If the mice which died early and were not at risk be omitted the incidence amounts to 14 per cent.

4. One of the oxidative derivatives of cholesterol reported by Fieser and Bischoff to be carcinogenic has given positive results when tested in the writer's laboratory. The most active of their compounds, 6β-hydroperoxy-Δ^4-cholestene-3-one, which is reported by Bischoff to give 60 per cent sarcomas in Buffalo mice, has induced sarcoma in 4 out of 50 treated C_{57} mice.

5. The degree of response to cholesterol as carcinogen has been found to fluctuate widely. It is suggested that such variations of sensitiveness are revealed by carcinogenic stimuli of low-intensity, and are linked with congenital and environmental factors.

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DIGESTIBILITY OF CERTAIN HIGHER SATURATED FATTY ACIDS AND TRIGLYCERIDES

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INTRODUCTION

In view of the rather wide differences which the writers ('40) have found between the digestive coefficients of certain fats, it has seemed desirable to determine the digestibility of several pure saturated fatty acids and the corresponding triglycerides. Digestion tests have been conducted with mature male rats with diets containing different percentages of pure stearic, palmitic, myristic, and lauric acids and with pure tristearin, triplamitin, trimyristin, and trilaurin.

PREVIOUS INVESTIGATIONS

In experiments with dogs, Arnschink (1890) found tristearin to be 9–14% digestible. Nearly all of the undigested stearin was present in the feces as the triglyceride. Lyman ('17) obtained digestive coefficients of approximately 95% for glyceryl palmitate and 82% for palmitic acid. He states that the melting point of an ester is not the only, and probably not the chief, factor determining the rate of hydrolysis and absorption.

MATERIAL AND METHODS

Stearic, palmitic, myristic, and lauric acids and the corresponding triglycerides, each of the highest purity obtainable, were purchased from a firm specializing in pure organic chemicals. Pure virgin olive oil was also purchased. The oil was stored at about 4°C.

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The percentage composition of the diets which were fed to the rats was as follows: Diet 1 — casein 18.6; salts 4.0; dextrose 75.4; yeast vitamins 2.0; and no fat. Diet 2 — casein 20.0; salts 4.3; dextrose 68.7; yeast vitamins 2.0; and fat 5.0. To 1 kilo of each diet was added 100 ml. of cod liver oil concentrate.[1]

EXPERIMENTAL PROCEDURE

Each diet containing 5% of a mixture of olive oil and a definite proportion of a fatty acid or triglyceride was fed to eight mature male rats for 10 days. After a preliminary period of 3 days, the feces were collected for 7 days from each rat and the quantity of feed consumed by each was determined. Each rat was then changed to a fat-free diet and, after a preliminary period of 3 days, the feces were collected for 7 days. The feces were dried to constant weight at 100°C. and the fat content was determined as previously described by the writers ('42). When the diet contained fatty acids, the digestive coefficient was calculated as the percentage of the total fatty acids. The digestive coefficient was calculated for each of the eight rats on each diet, but only the average value is reported.

EXPERIMENTAL RESULTS

The results of the digestion tests with two lots of olive oil and with mixtures of olive oil and different proportions of stearic, myristic, and lauric acids are shown in table 1. The melting points of the pure fatty acids and of the mixtures are shown also. Two lots of olive oil were used, as the first was insufficient.

The results of the tests with the mixtures of olive oil and saturated fatty acids show that stearic acid depressed the digestibility of the mixture only slightly less than the proportion of stearic acid present. The digestive coefficients for stearic acid alone have been calculated on the assumption that the reduction in digestibility was due entirely to unabsorbed stearic

[1] 100 ml. of an ethereal extract from saponified cod liver oil was added to 1 kg of the diet and the mixture was agitated until the ether had evaporated.

TABLE 1

Digestibility by male rats of mixtures of certain saturated fatty acids and olive oil when the diets contained 5% of fat.

DESCRIPTION OF FAT MIXTURE	MELTING POINT OF PURE FATTY ACIDS	MELTING POINT OF MIXTURE	DIGESTIVE COEFFICIENT OF MIXTURE		CHANGE IN DIGESTIBILITY COMPARED WITH OLIVE OIL	CALCULATED DIGESTIBILITY OF PURE FATTY ACIDS
			Average [1]	Range		
	°C.	°C.	%	%	%	%
Olive oil no. 4085 [2]			97.4	94.1– 99.3		
Olive oil no. 4095			99.5	99.1–100.0		
Stearic acid 5%, olive oil 95%	69	43	92.6	90.5– 95.5	– 4.8	9.4
Palmitic acid 5%, olive oil 95%	63	37	94.2	91.9– 96.6	– 3.2	39.6
Myristic acid 5%, olive oil 95%	53		99.1	97.6–100.0	+ 1.7	
Lauric acid 5%, olive oil 95%	44		98.0	97.3– 99.0	+ 0.6	
Stearic acid 10%, olive oil 90%		51	88.3	85.0– 91.2	– 9.1	13.3
Palmitic acid 10%, olive oil 90%		44	90.8	87.6– 94.2	– 6.6	37.1
Myristic acid 10%, olive oil 90%		32	97.6	95.0– 99.0	+ 0.2	
Lauric acid 10%, olive oil 90%			98.3	97.1– 99.7	+ 0.9	
Stearic acid 15%, olive oil 85%		55	85.0	79.4– 89.5	– 12.4	21.0
Palmitic acid 15%, olive oil 85%		48	86.6	82.4– 89.8	– 10.8	31.2
Myristic acid 15%, olive oil 85%		39	97.1	93.8–100.0	– 0.3	
Lauric acid 15%, olive oil 85%			97.2	96.6– 98.5	– 0.2	
Stearic acid 25%, olive oil 75%		59	76.5	71.1– 80.1	– 20.9	19.6
Palmitic acid 25%, olive oil 75%		53	77.6	75.0– 82.4	– 19.8	23.8
Myristic acid 25%, olive oil 75%		44	92.7	88.4– 97.0	– 4.7	81.9
Lauric acid 25%, olive oil 75%		32	92.6	82.5– 96.3	– 4.8	81.5
Stearic, palmitic, myristic, and lauric acids, 2.5% each; olive oil 90%			93.5	90.4– 97.0	– 6.0	

[1] Each digestive coefficient represents the average value obtained with eight rats.

[2] Olive oil no. 4085 was used in all mixtures except the last one.

acid. To obtain these figures, the percentage of fatty acid in each mixture was calculated as the percentage of the total fatty acids, since the digestive coefficients were calculated on that basis.

The results of the digestion tests with the mixtures of palmitic acid and olive oil show that each mixture had a somewhat higher digestive coefficient than a similar mixture of stearic acid and olive oil. The approximate digestive coefficients for palmitic acid alone were calculated in the same manner as for stearic acid.

Myristic and lauric acids were very thoroughly absorbed when the percentage of acid in the mixture did not exceed 15%; but when the proportion was increased to 25%, the digestibility was materially lower. The approximate digestive coefficients for myristic and lauric acids alone are shown only for the highest level of intake.

The digestibility of the mixture of 2.5% each of stearic, palmitic, myristic, and lauric acids and olive oil no. 4095 was lower than might have been expected from the results previously reported in table 1. This mixture had a digestive coefficient six points lower than the olive oil used although the mixture contained only 2.5% each of stearic and palmitic acids.

The results of the digestion tests with the mixtures of triglycerides and olive oil are shown in table 2. The results with tristearin show that this triglyceride, whether present singly or together with other triglycerides in a mixture with olive oil, depressed the digestibility of the fat nearly as much as the percentage of tristearin present. When the mixtures contained 5 and 10% of tristearin, the approximate digestive coefficients of the tristearin alone were calculated on the assumption that the reduction in digestibility was due entirely to unabsorbed tristearin.

The results of the tests with the mixtures containing tripalmitin indicate that this triglyceride was much more digestible than tristearin. The approximate digestive coefficients for tripalmitin alone, when the mixtures contained 5 and 10% of this triglyceride, are shown.

TABLE 2

Digestibility by male rats of mixtures of certain saturated triglycerides and olive oil when the diets contained 5% of fat.

DESCRIPTION OF FAT MIXTURE	MELTING POINT OF TRIGLYCERIDE	MELTING POINT OF MIXTURE	DIGESTIVE COEFFICIENT OF MIXTURE		CHANGE IN DIGESTIBILITY COMPARED WITH OLIVE OIL	CALCULATED DIGESTIBILITY OF TRIGLYCERIDE
			Average[1]	Range		
	°C.	°C.	%	%	%	%
Olive oil no. 4085[2]			97.4	94.1– 99.3		
Olive oil no. 4095			99.5	99.1–100.0		
Tristearin 5%, olive oil 95%	70	55	92.7	91.5– 94.4	— 4.7	6.0
Tripalmitin 5%, olive oil 95%	65	46	95.8	91.2– 99.3	— 1.6	84.0
Trimyristin 5%, olive oil 95%	56		96.4	93.5– 99.8	— 1.0	
Trilaurin 5%, olive oil 95%	48		98.3	94.8– 99.9	+ 0.8	
Tristearin 10%, olive oil 90%		59	89.2	88.0– 91.3	— 8.2	8.0
Tripalmitin 10%, olive oil 90%		51	95.6	92.9– 97.2	— 1.8	82.0
Trimyristin 10%, olive oil 90%		42	99.0	97.8–100.0	+ 1.6	
Trilaurin 10%, olive oil 90%			99.9	99.5–100.0	+ 2.5	
Tristearin 5%, trimyristin 5%, olive oil 90%		54	95.3	93.9– 96.8	— 4.2	
Tristearin 5%, trilaurin 5%, olive oil 90%		53	95.2	91.1– 96.6	— 4.3	
Tripalmitin 5%, trimyristin 5%, olive oil 90%		42	96.3	93.7– 98.4	— 3.2	
Tripalmitin 5%, trilaurin 5%, olive oil 90%		40	97.7	96.6– 98.5	— 1.8	
Tristearin, tripalmitin, trimyristin, and trilaurin 2.5% each; olive oil 90%			96.9	94.1– 98.6	— 2.6	

[1] Each digestive coefficient represents the average value obtained with eight rats.

[2] Olive oil no. 4085 was used in the first two series of digestion tests and sample no. 4095 was used in the third series.

The mixtures containing 5 or 10% each of trimyristin or trilaurin were as digestible as the olive oil alone. Under these conditions each triglyceride was practically 100% digestible.

A comparison between the results obtained with the fatty acids and those with the corresponding triglycerides indicates that both tristearin and stearic acid were very poorly absorbed, whereas tripalmitin appeared to have been much better absorbed than palmitic acid. Myristic and lauric acids and the corresponding triglycerides were utilized equally well.

SUMMARY

Experiments were conducted with mature male rats to determine the digestibility of pure stearic, palmitic, myristic and lauric acids and of the corresponding triglycerides. Each fatty acid was mixed in the proportions of 5, 10, 15, and 25% with pure olive oil, and each triglyceride was mixed in the proportions of 5 and 10%. The fat mixture constituted 5% of the diet. The following results were obtained:

Stearic acid was very poorly absorbed at each level of intake, the approximate digestive coefficients ranging from 9.4 to 21%.

Palmitic acid was utilized somewhat more efficiently than stearic acid, the approximate digestive coefficients ranging from 23.8 to 39.6%.

Myristic and lauric acids were practically 100% digestible when the fat mixtures contained 5, 10, or 15% of either acid, but the digestibility was somewhat lower when the mixture contained 25% of acid.

Tristearin was very poorly utilized, the approximate digestive coefficients being 6 and 8% when the fat mixtures contained 5 and 10% of the triglyceride.

Tripalmitin was much more digestible than tristearin, the approximate digestive coefficients being 84 and 82% when the fat mixtures contained 5 and 10% of the triglyceride.

Trimyristin and trilaurin were very thoroughly absorbed.

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Effects of Dietary Free C_{18} Saturated and Unsaturated Fatty Acids and DL-Methionine Choline Chloride Mixture on Plasma Cholesterol of the Male Albino Rat

By Erwin T. Janssen

The relationship of dietary fatty acids and the cholesterol levels found in serum and liver is, and has been, under study by many investigators. The relationships involved are undoubtedly intricate and more complex than most investigators seem to imply. Although Jones *et al.*[1] imply a close relationship between rats and man in dietary requirements and habits, it is good to keep in mind differences that are intrinsic to both man and experimental animals. Effects produced on experimental animals and those produced upon man in dietary studies in some cases may be vastly different. With this in mind, an attempt will be made throughout the following paper to limit discussion to results found through work with experimental animals.

Aftergood and her co-workers state that many investigators believe that serum cholesterol levels are controlled by fat intake regardless of the source of the fat.[2] However, she points out that evidence exists to show that the source of fat may be vitally concerned with the resulting cholesterol level.

In a majority of cases, fats have been fed in the form of triglycerides. These have been arbitrarily classified into two groups: animal fats and vegetable fats. In both the animal and vegetable fats the majority of fatty acids are found as the triglycerides, and only a small fraction are found as the free acid.[3] Recently, Malmros and Wigand have shown evidence that indicates a better classification might be based on the degree of saturation, rather than on the source of the fat.[4]

Various investigators have shown that the feeding of vegetable fat gives a lower serum cholesterol level than that which is found if animal fat is administered. This might indicate that the unsaturation of the vegetable fats plays a part in the lower cholesterol levels. However, other components present in the fats cannot be disregarded. In a study performed on rabbits, Swell *et al.*[5] show that the blood cholesterol level is greatest with soybean oils that have the highest

iodine number. Also, if a 2% cholesterol diet containing free acids was fed, the free acids were more effective in raising the blood cholesterol level than were the neutral fats. Of the free fats tested, oleic acid was the most effective in raising the cholesterol values, followed by stearic and linoleic acids.

The role of choline and methionine as methyl donors and their relationship to plasma and liver cholesterol were deemed worthy of investigation. It was thought possible that some effect upon the cholesterol content of plasma and liver might be manifested by dietary addition of choline or methionine. Jones *et al.*[1] found that in rats fed a supplement of 0.5% DL-methionine, a rise in the serum cholesterol value took place. However, they point out that Mann and co-workers noted just the opposite effect in the serum of methionine deficient monkeys made deficient by feeding soybean protein when a methionine supplement was given.

Since in a majority of studies the fats have been administered as triglycerides, the following study was designed to investigate the effects of dietary fats as free fatty acids on both plasma and liver cholesterol. In addition, diets containing supplements of DL-methionine and choline chloride were fed to determine if there was any effect on plasma and liver cholesterol.

Experimental

A group of twenty-five male albino rats of the Holtzman strain was divided into five groups of four animals each and one group of five animals, this group to be used as the control group. The animals were housed in screen bottom animal cages of the Army Medical School type and maintained on food and water *ad libitum*. The room in which they were kept was held at a temperature of around 25° C., but fluctuations of 3° C. did occur. The animals were maintained on Rockland Rat Diet for a period of approximately seven weeks before dietary studies were begun. Records of daily diet consumption were kept, and body weights were recorded at two day intervals.

The basic experimental diet constituent was Rockland Rat pellets. At first, all animals were maintained on the diet in pellet form. When the experimental diets were made up, the pellets were ground to powder form and the various dietary supplements were then blended in with an electric mixer. The dietary constituents and their relative amounts are given in Table 1. The diets containing the unsaturated linoleic acid were stored under refrigeration. All diet supplements used were obtained from the Matheson, Coleman & Bell Division of the Matheson Company. The choline chloride was added at the level of 0.350 g. per 100 g. of diet. This supplemented the 0.157 g. stated by the manufacturer to be present in the diet to a level of 0.502 g. per 100 g. of the stock diet. The DL-methionine was added in the

Table 1

Experimental Diets used. The DL methionine was added in amounts of 0.5 g. per 100 g. of diet. The choline chloride was added in the amount of 0.350 g. per 100 g. of diet. This gave a concentration of 0.502 g. per 100 g. of diet of choline chloride. (0.152 g. per 100 g. diet present as stated by manufacturer.) The combined total gave approximately a 1.0% supplement.

Group	Stearic Acid	Linoleic Acid	Stock Diet	DL-methionine Choline Chloride
A	15 g.	——	85 g.	——
B	15 g.	——	85 g.	1.0 g.
C	——	15 g.	85 g.	——
D	——	15 g.	85 g.	1.0 g.
E	——	——	100 g.	——
F	——	——	100 g.	1.0 g.

amount of 0.5 g. per 100 g. of stock diet. To make the 15% free fatty acid diet, 15 g. of the acid was added to 85 g. of the stock diet. The diets were mixed in quantities sufficient for approximately ten day periods. Frequent mixing was done in an effort to prevent any change in the diet composition. It should be noted that the 15% fatty acid amounts were added to the stock diet which already contained about 5% fat.

The six cages of animals were divided into pairs—Group A and B, Group C and D, Group E and F. Both Groups A and B were fed 15% stearic acid, Group B in addition receiving the methionine-choline supplement. Groups C and D were fed the 15% linoleic acid, and Group D received the supplement. In Groups E and F, the stock diet was fed, Group F receiving the added methionine-choline supplement.

The thirteen-week-old animals were started on the diets, and at two week intervals 1.5 to 2.0 ml. of blood were collected from each animal under ether anesthesia by bleeding the tail vein. One to two drops of a sodium oxalate solution were added as an anticoagulant, and the formed elements were separated from the plasma by centrifugation. Separate samples were taken from the supernatant plasma for the determination of free and total cholesterol. In every analysis except the final one, 0.3 ml. of the plasma was used for the determination of the free cholesterol. For the last analysis 0.4 ml. was used. Throughout the entire analysis, 0.2 ml. of plasma was used for the determination of total cholesterol. Both free and total cholesterol were analyzed by the method of Sperry and Webb.[6]

After eight weeks on the diets, the animals were sacrificed by heart puncture under ether anesthesia. The livers were removed, blotted dry, and weighed to the nearest 0.1 gram. Approximately 0.5 g. of liver was taken for analysis and this portion was weighed accurately to the nearest milligram. The liver sample was homogenized in 4 ml. of 1:1 solution of ethyl alcohol and acetone, and the heated 4

boiling. The homogenate was extracted twice, and the combined extracts were made up to a volume of 10 ml. Both free and total liver cholesterol were each determined in 2 ml. aliquots by the method of Sperry and Webb. Standards containing 0.1 mg. of cholesterol were used with each group of determinations.

Discussion of Results

It is evident from Table 2 that the gain in weight for all of the groups is nearly identical. Table 3 shows the daily average diet intake per rat. A possible explanation for the higher values seen in the data for the stearic acid diets is that this solid acid is not as readily absorbed into the intestine as is the liquid linoleic acid.

Table 2

Animal Weight Gain During Experiment. The Results Are the Average Per Animal in Each Cage

Group	Initial weight (g.)	Final weight (g.)	Gain (g.)
A	356	425	69
B	365	434	69
C	380	460	80
D	375	455	80
E	343	419	76
F	362	424	62

Table 3

Daily Average Intake Per Rat During Experimental Period

Group		Daily average per rat (g.)
15% Stearic	(A)	24.5
15% Stearic + M-C*	(B)	25.0
15% Linoleic	(C)	20.6
15% Linoleic + M-C*	(D)	19.6
Stock Diet	(E)	22.6
Stock Diet +M-C*	(F)	22.5

*Signifies the DL-methionine and choline chloride supplement.

The overall plasma cholesterol levels for the groups on stearic acid, linoleic acid, and stock diet are shown in Table 4. It is to be noted that there is no significant difference between the animals fed the 15% stearic acid diets and the animals fed the regular stock diets. However, a significant difference ($p < 0.02$) was seen in the animals fed the 15% linoleic acid. Thus, the cholesterol level of the rat is raised by feeding of the unsaturated free fatty acid. This is in agreement with the work done by Klein on acids fed as the triglycerides.[7]

Table 4

Overall Total Cholesterol Levels of Plasma. Results Are Expressed in mg. per 100 ml. Plasma (mg.%)

Groups		Total Cholesterol of Plasma (mg.%)	(p) different from stock*
15% Stearic	(A & B)	42.1	>0.05
15% Linoleic	(C & D)	52.9	0.02
Stock Diet	(E & F)	39.7	—

*Probability greater than 0.05 is taken to be non-significant.

Table 5

Total Cholesterol of Plasma for Individual Groups. The Free Cholesterol Values Were Not Significant

Group		Total Cholesterol of Plasma (mg.%)	(p) of difference between groups*
15% Stearic	(A)	41.7	
15% Stearic +M-C	(B)	42.4	>0.05
15% Linoleic	(C)	48.1	
15% Linoleic +M-C	(D)	57.7	0.05>p>0.02
Stock Diet	(E)	36.5	
Stock Diet +M-C	(F)	42.4	>0.05

*Probability greater than 0.05 is taken to be non-significant.

Table 5 shows the results of feeding the methionine-choline supplement. No significant difference was noted in the free cholesterol in any of the groups. Likewise, the total cholesterol values of Groups A and B and Groups E and F showed no significant difference. However, in the groups receiving the 15% linoleic acid (Groups C and D) the methionine-choline supplement produced a significantly higher total plasma cholesterol level than was produced if only 15% linoleic acid was fed. It will also be noted that the lowest level in the 15% linoleic acid groups (Group C) still remained above the values of the 15% stearic or the stock diet groups. The higher values of the methionine-choline supplemented animals are in agreement with the findings of Jones *et al.*[1]

Table 6 shows the liver cholesterol values. In comparing the ester values of the groups with the stock diet group it is seen that the only significant difference is to be found in the animals on the 15% linoleic acid. If the plain 15% fat and stock diets are to be compared to their corresponding methionine-choline supplemented groups, only the 15% linoleic acid groups differ significantly. The 15% linoleic diet with the supplement showed a lower liver cholesterol ester level than its plain 15% linoleic acid counterpart ($p = 0.02$).

Friedman and Byers have cited that the liver is the chief organ

of regulation of ester cholesterol.[8] Thus, above results might tend to indicate that the livers of the animals on the 15% linoleic acid diet in the absence of a methionine-choline supplement (Group C) were more effective in controlling the plasma cholesterol. More work would be needed in order to arrive at a definite conclusion about this relationship.

Table 6

Liver Cholesterol After Eight Weeks. The Comparison Is Made Using the Cholesterol Ester Values

Group	Free Chol. (mg.%)	Total Chol. (mg.%)	Ester Chol. (mg.%)	(p) diff. than stock*	(p) diff. between groups*
A	165	194	29	>0.05	>0.05
B	162	197	35	>0.05	
C	190	313	123	0.02	0.02
D	170	234	64	0.02	
E	197	226	29	—	>0.05
F	182	213	31	>0.05	

*Probability greater than 0.05 is taken to be non-significant

Summary

Five groups of four animals per group were maintained on separate diets composed of 15% stearic acid, 15% linoleic acid; and three groups had diets supplemented with approximately a 1.0% combined mixture of DL-methionine and choline chloride. Blood was drawn at two week intervals and analyzed for both total and free cholesterol. At the completion of the eight week period, the animals were sacrificed by heart puncture. The livers as well as the plasma were analyzed for the cholesterol content. The results indicated that the feeding of 15% free acids caused a rise in the plasma cholesterol levels, the significant rise being in animals on the 15% linoleic acid diet. When a supplement of DL-methionine and choline chloride was fed, it produced a greater rise in the cholesterol levels than was produced by only the 15% linoleic acid. The liver cholesterol level showed only slight variations from the values of the control group, except in the case of the animals fed the 15% linoleic acid. The unsupplemented 15% acid diet showed a higher liver cholesterol ester content than the diet containing added methionine-choline. Indications were that on a 15% linoleic acid diet the addition of methionine-choline decreases the ester content of the liver while raising the plasma cholesterol ester content.

ACKNOWLEDGMENTS

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Jour. Appl. Physiol. 11(1): 8-11, 1957

Effect of Palmitic and Stearic Acid on Cholesterol Absorption in Man[1]

E. KARVINEN, T. M. LIN AND A. C. IVY. [illegible] *Department of* [illegible] *University of Illinois* [illegible] *Illinois*

ABSTRACT

KARVINEN, E., T. M. LIN AND A. C. IVY. *Effect of palmitic and stearic acid on cholesterol absorption in man.* J. Appl. Physiol. 11(1): 8-11. 1957. — This study on human subjects was made because it had [illegible] rats that stearic and palmitic acid [illegible] inhibited the intestinal absorption of cholesterol. Fifteen [illegible] healthy adult [illegible] were fed a basal diet containing [illegible] 125 gm of protein and [illegible] carbohydrate daily. The fecal excretion of cholesterol and total lipid was determined during the [illegible] days of eight[illegible] day dietary periods. Nine subjects consumed the basal diet [illegible], then the basal diet plus [illegible] gm/day of palmitic acid, then the basal diet plus one egg per day [illegible] cholesterol [illegible] plus 30 gm of palmitic acid. Six [illegible] routine except that 30 gm of stearic acid replaced the palmitic acid [illegible] gm of cholesterol were fed [illegible] eggs. The absorption of the endogenous and the dietary cholesterol was not influenced significantly by either fatty acid. The palmitic and the stearic acid were absorbed by the human subject [illegible] and [illegible]%, respectively.

It has been repeatedly observed by us in balance studies on rats that palmitic and stearic acid inhibit the intestinal absorption of both dietary [illegible] and endogenous [illegible] cholesterol. Furthermore, Swell and [illegible] have reported that the feeding of stearic acid to rats was followed by a decline in blood cholesterol. [illegible], using small rats of a different strain in groups of four or five, noted that stearic acid, in contrast to oleic acid, did not facilitate the absorption of dietary cholesterol when added to an otherwise fat-free diet. [illegible] acid and other long chain fatty acids have been found by [illegible] and Noble [illegible] to increase cholesterol excretion. Therefore, the present study was undertaken to evaluate in man the efficacy of palmitic and stearic acids, as representative of the saturated, high melting point fatty acids, for [illegible] absorption, in view of the practical implication of a food additive which might deplete the intestinal cholesterol pool.

EXPERIMENTAL

Diet. The diet was the same as that described [illegible] [illegible]

[illegible]

Received for publication [illegible]
[1] This work was [illegible] States Air Force [illegible] Research [illegible] Air Research and Development Command [illegible]

Subjects. [illegible]

experiment with palmitic acid, and another group of six subjects served in the experiment on stearic acid. One additional subject started the experiment but became indisposed. The subjects were young healthy adults.

Design of Experiments. In the case of palmitic acid the control or basal diet (BD) was fed for 7 days; then, 30 gm/day of palmitic acid was added for 7 days; then, one egg (300 mg cholesterol) was added daily without the palmitic acid for 7 days and then with the palmitic acid for 7 days (table 1A).

In the case of stearic acid, the basal diet was fed for 7 days; then, 30 gm of stearic acid was added daily for 7 days; then, 3 gm of cholesterol was added daily for 7 days without the stearic acid and then with the 30 gm/day of stearic acid for 7 days.

Each test week started Monday at breakfast and ended the following Monday. A warming market was given Thursday with breakfast and Sunday with breakfast. Fecal collections were made daily from the 4th through the 6th day. The chemical analyses (8) were made on the pooled daily specimens of each subject, the portion between the breakfast being used. Blood for serum cholesterol determination was collected on Sunday morning.

RESULTS

Effect of Palmitic Acid on Absorption of Cholesterol. The excretion of lipids when one egg was added to the basal diet with or without palmitic acid is shown in table 1. Inspection of the results shows that the addition of the egg (300 mg of cholesterol) daily increased the sterol excretion as though no cholesterol had been absorbed (table 1A). Such a conclusion is not warranted, however, because of the size of the standard deviations of the means of *periods* 2 and 3.

Palmitic acid had no significant effect on the excretion of endogenous cholesterol or on the absorption of added cholesterol (table 1A).

Effect of Stearic Acid on Absorption of Cholesterol. The excretion of lipids when 3 gm of cholesterol was added to the basal diet with and without stearic acid is shown in table 1B. When 3 gm of cholesterol was the only addition 55% of it was absorbed. Incorporation of the stearic acid in the diet was associated with a decrease in the absorption of dietary cholesterol to 47%, a difference that was not significant (table 1B). The excretion of nonsterol unsaponifiable material was increased, but not significantly, when both cholesterol and stearic acid were included in the diet (*period* 3).

Stearic acid had no significant effect on the excretion of endogenous cholesterol (table 1B). Thus, stearic acid failed to deplete intestinal cholesterol in man, although in both trials an insignificant increase in sterol elimination was noted.

Utilization of Palmitic and Stearic Acid by Man. When fed 30 gm daily amounts, the utilization of palmitic acid amounted to an average of 55%, and of stearic acid to an average of 21%.

Serum Cholesterol. One week on any dietary regimen employed was not enough to produce significant changes in the serum cholesterol level.[2]

DISCUSSION

First, the result in table 1A, *period* 3, where one egg or 300 mg of cholesterol was given, suggesting that all of the 300 mg of cholesterol added was excreted, indicates that the sensitivity of the method is not sufficient to study percentage absorption of quantities of cholesterol as small as 300 mg.

Because the 30 gm of palmitic acid had no significant effects on the absorption of endogenous cholesterol, because approximately 55% of the 30 gm of palmitic acid was utilized, and because it was impractical to add more palmitic acid to the diet, a larger quantity of cholesterol was not tested with palmitic acid. Instead, it was decided to use stearic acid with 3 gm of cholesterol daily because studies [illegible] indicated that stearic acid might not be utilized as well as palmitic acid, but 30 gm of stearic acid did not significantly impair cholesterol absorption.

The failure in these experiments [illegible] to confirm our observations on rats regarding the impairment of cholesterol absorption by feeding palmitic and stearic acid may be due to the relatively smaller amounts of these acids fed to the human subjects [illegible] that an acceptable human diet has to [illegible] some low melting point [illegible]

[2] Additional tables [illegible] as document [illegible] with the [illegible] Project, Protection [illegible] Washington 25, D.C. [illegible] the document [illegible] with [illegible] $1.25 for [illegible] Make checks or [illegible] Photo [illegible]

TABLE 1. EXCRETION OF LIPIDS IN PALMITIC ACID AND STEARIC ACID EXPERIMENTS

Period	Diet	No. of Subj.	Total Lipid, gm/day	Total Lipid Minus Soap, gm/day	Unsaponifiable gm/day	Sterol, mg/day	Cholest. Absorp. mg[b]	Cholest. Absorp. %[c]
				A. Palmitic acid experiments				
1	BD	9	6.17±1.69	4.13±1.2	1.42±0.39	908±278[b]		
2	BD + palmitic acid 30 gm/day	9	19.81±2.77	11.11±3.67	1.36±0.28	872±[illegible][b]		
3	BD + egg[e]	9	6.05±2.33	5.30±1.73	1.96±0.44	1212±378	0[d]	0
4	BD + egg[e] + palmitic acid 30 gm/day	9	19.36±2.00	6.39±1.00	1.40±0.38	103±230	69[d]	[illegible]
				B. Stearic acid experiments				
5	BD	6	5.50±1.21	3.69±0.42	1.43±0.21	844±322[a]		
6	BD + stearic acid 30 gm/day	6	30.61±5.43	20.27±3.17	2.86±0.09	930±228[g]		
7	BD + 3 gm. cholesterol	6		5.41±1.32	2.04±0.25	120±140	1715[h]	[illegible]
8	BD + 3 gm. cholesterol + stearic acid 30 gm/day	6		27.97±8.89	5.28±1.60	518±421	1412	47.0

BD = basal diet. [a]*Period* 7, 300 − (1212 − 908) = 0; *period* 4, 300 − (1103 − 878) = 69; *period* 5, 3000 − (2129 − 824) = 1715; *period* 8, 3000 − (2518 − 930) = 1412. [b]*Period* 1 vs. *period* 2; t = 0.51. [c]300 mg of sterol per day. [d]*Period* 3 vs. *period* 4; t = 0.63. [e]% of intake: $100 - \frac{100}{3000} \times (\text{Sterol}_{\text{diet}} - \text{Sterol}_{\text{fat}})$. [g]*Period* 5 vs. *period* 6; t = 1.05. [h]*Period* 7 vs. *period* 8; t = 1.30.

stearic acid. These fatty acids were fed to the rats at a level of 7.6%. As indicated above, to include them at this level in the human menu was impractical because of the general unacceptability of the diet.

Palmitic acid (30 gm) when fed to human subjects at a level of 4.5% in a diet containing an additional 20 gm of other fat (this fat was present in food items to the extent of 3.2% by dry weight which, with the palmitic acid, made up 7.6% by dry weight of the diet) was utilized to the extent of 55%.

A review of the literature did not reveal that the utilization of palmitic acid by human beings when given as the only food lipid had been studied. In dogs palmitic acid was found to be utilized to an extent of 82% (9), but in the rat Cheng *et al.* (12) and we (2, 3) found a 10% utilization. When fed with olive oil in relatively small amounts to rats by Hoag and and Snider (10) from 22 to 40% was utilized.

Stearic acid (30 gm) when fed to our human subjects at a level of 4.5% in the same basal diet as the palmitic acid was fed, was utilized to the extent of 21%.

A review of the literature did not reveal that the utilization of stearic acid by human subjects had been determined. However, in dogs it has been reported that tristearin is only 9–14% utilized (11). Cheng *et al.* (12) found stearic acid to be utilized to approximately 14% in rats. When fed in relatively small amounts with a large proportion of olive oil to rats, 9–21% of stearic acid was utilized (10). On analyzing the utilization of various triglycerides by adult human subjects, Mattil (13) concluded that stearic acid appears to be utilized better by human beings than by rats. Our results confirm this conclusion. Thus the failure of palmitic and stearic acid to impair cholesterol absorption in man may be explained as due to their better utilization by man than by the rat.

We express our thanks to Dr. Norris Emerson of the Distillation Products Company, Rochester, N.Y., to Dr. K. E. Mattil and Dr. Herbert Fishbein of Swift and Co., Chicago, for assisting us in the preparation of various products used in this study, also to the individuals who served as subjects.

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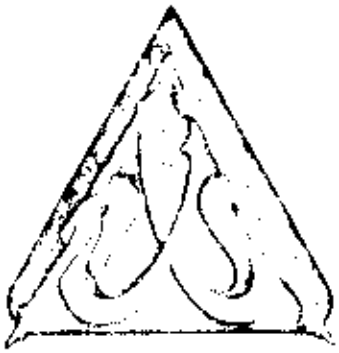

Methods, apparatus: new product research, process development and d[illegible]

A rapid gas chromatographic method for the determination of fatty acid compositions of [illegible] and castor oil

by A. Kato and Y. Yamuni [illegible]
Government Chemical Industrial Research Institute, Tokyo, Japan

Gas-liquid chromatography is a convenient method for the determination of fatty acid compositions of oils and fats. Oils and fats are transesterified,[1] and the methyl esters analysed by gas-liquid chromatography. The method is routine, but transesterification takes an hour or more. Davidson and Dutton[3] have developed a rapid analytical microtechnique for gas chromatographic determination of the fatty acid composition of vegetable oils. Their method uses a microreactor for transesterification of triglyceride.

A glass technique has been developed with rapid transesterification of oils and fats. The method involves using a precolumn for the preparation of methyl esters from glycerides. A glass column (0·4 × 187cm), consisting of a precolumn and column sections is used (Fig. 1).

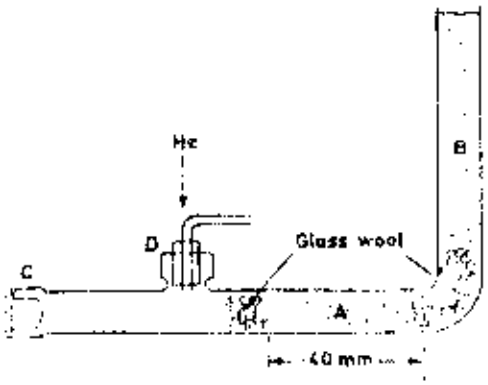

Fig. 1. A precolumn prepared in a Pyrex glass column; A = 50 per cent sodium hydroxide on Chromosorb W AW 60-80 mesh; B = 25 per cent diethyleneglycol succinate on Chromosorb W AW 60-80 mesh; 4 × 30mm; C = Sample inlet; D Helium (carrier gas) inlet.

Chromosorb W AW 60-80 mesh was mixed with a solution of sodium hydroxide or potassium hydroxide (10g) in distilled water (10ml), and the dry mixture was inserted into a precolumn (0·4 × 4cm). The remainder of the column (0·4 × 183cm) was packed with 25 per cent diethylene glycol succinate on Chromosorb W AW 60-80 mesh for soybean oil and other oils and fats, and with [illegible] per cent silicon SE-30 on Chromosorb W AW 100-120 mesh for castor oil. The gas chromatograph was a Shimadzu 1C with a flame ionisation detector. Temperatures for the precolumn injection port and column were [illegible] oil and 200°C for other oils and fats. The [illegible] helium was 40ml/min; for hydrogen [illegible] compressed air, 1kg/cm². The chart speed was [illegible] 4 d of a solution of soybean oil (2g) in [illegible] (1:1, 20ml), was injected. In the case of [illegible] a solution of castor oil (2g) in methanol (20) [illegible]

The results of the determination of fatty acid [illegible] of soybean oil by this method were in [illegible] analytical results of routine methods (see Table).

Table
Fatty acid composition of soybean oil

Columns	Palmitic	Stearic	Acids % Oleic	Linoleic	Linolenic
A*	11·4	2·5	24·9	[illegible]	[illegible]
B	11·1	2·9	21·8	[illegible]	1·8
C*	13·7	2·3	23·9	54·2	[illegible]

A = 10% NaOH + 25% DEGS (new method); B = [illegible] DEGS (new method); C = 25% DEGS (AOC [illegible]
* Mean value of two results.

A [illegible] separation of methyl ricin[illegible] was investigated by [illegible] but this [illegible] applicable to the determination of ricinol[illegible]

The 50 per cent sodium hydroxide [illegible] column was used for the rapid determ[illegible] in castor oil. The transesterification [illegible] carried out in a NaOH-column [illegible] esters were analysed in the [illegible] the chromatograph by [illegible] [illegible] 4 per cent. [illegible]

References
1 [illegible]
2 [illegible]
3 [illegible]
4 [illegible]

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46(11): 1695–1703, 1967

The Monoglyceride Pathway of Fat Absorption in Man *

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Abstract. The absorption of fat was studied in five male subjects with cannulation of the thoracic duct in the neck by the administration of doubly labeled monoglycerides, or triglyceride as well as labeled free glycerol or labeled free oleic acid, by gastric or duodenal intubation.

Total recoveries of the administered glyceride radioactivity from the lymph lipids ranged from 35 to 53% for the glycerol label (tritium) and from 35 to 57% for the fatty acid label (^{14}C). The recovery of administered radioactive free glycerol in lymph lipids was only 4.1%, even when given in mixture with bile salts, fatty acid, and monoglyceride.

A comparison of the isotope ratios of the two components (glycerol and fatty acid) of the lymph glycerides with the ratios of these components of the original meal glyceride showed little change during the initial period of fat absorption, indicating that the doubly labeled monoglycerides passed into the lymph intact. During the later part of the period of major fat absorption, the ratios in lymph lipids changed due to loss of glycerol representation, indicating monoglyceride hydrolysis and portal venous diversion of free glycerol.

Confirmation of the intact nature of 2-monoglyceride during absorption was made by analyzing the amount and position of the labeled fatty acid in the lymph triglycerides. The percentage of labeled fatty acid in the various positions of the lymph triglycerides was virtually identical with that of the meal during the initial period of fat absorption and then changed reflecting isomerization of fatty acids and subsequent complete hydrolysis of the glycerides.

The 2-monoglyceride pathway appears to be the major route of fat absorption for man during normal digestion and absorption of dietary triglyceride.

Introduction

The major portion of dietary fat is insoluble in water and important changes take place in the physicochemical state of dietary triglycerides in the gut lumen before absorption into the intestinal epithelium. Complete hydrolysis of triglyceride yields free fatty acids and glycerol; partial hydrolysis results in free fatty acids, monoglycerides, and diglycerides. Recently, it was shown in man that monoglycerides, free fatty acids, and conjugated bile salts readily form ultrafine aggregates or micellar solutions, from which fatty acids and monoglycerides may be absorbed into the intestinal mucosal cells (2). In whole animal studies, in which the glycerol and fatty acid moieties of glycerides were labeled with different isotopes, it was demonstrated (3–5) that 2-monoglycerides remain largely intact during intestinal absorption and reesterification into chylomicron triglycerides. This

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direct acylation pathway of monoglycerides to higher glycerides appears to be uniquely active in intestinal tissue, and is not appreciably demonstrable in other sites of triglycerides synthesis, such as the liver (6).

The present studies were undertaken to investigate glyceride absorption in man by administering singly and doubly labeled lipid compounds and analyzing in the subsequent hours lymph obtained by thoracic duct cannulation. The particular goal was to determine whether the monoglyceride pathway that has been shown in animals is also present in man and to attempt to assess its quantitative importance during normal fat absorption. The results of these studies suggest that the monoglyceride pathway is the predominant one during normal fat absorption.

Methods

Materials. The test glycerides were 2-^{3}H-glycerol-monostearate-1-^{14}C in which the stearic acid was in the 1- or 2-position and 2-^{3}H-glycerol-1,3-dioleate-2-oleate-1-^{14}C. The monostearin was synthesized from stearic acid-1-^{14}C and glycerol-2-^{3}H obtained from New England Nuclear Corp., Boston, Mass. Carrier stearic acid (Applied Science Laboratories Inc., State College, Pa.) was 99.8% pure (gas-liquid chromatography) and glycerol (Merck) was freshly redistilled at 2 mm Hg pressure. The method of preparation of 2-monostearin has been previously described (7). The final product had a specific activity of 30 μc/mg for ^{3}H and 15 μc/mg for ^{14}C. On hydroxylapatite thin-layer chromatography it moved as a single spot from which 98% of the radioactivity could be recovered. Saponification of the eluted material from the spot and partition between petroleum ether and acidified water phases showed that all the measurable ^{14}C activity was in the fatty acid and the ^{3}H in the glycerol moiety. The doubly labeled 2-monostearin was stored dry at −20°C but nevertheless showed gradual isomerization spontaneously to the 1-isomer. At the time of use in Study I, the material assayed 70% 2-monostearin, 30% 1-monostearin. The preparation of the micellar solution in Study II involved warming and some alkalinization, which appeared to enhance the isomerization so that assay of the final product in bile showed 20% 2-monostearin and 80% 1-monostearin.

The labeled triolein was prepared from oleic acid-1-^{14}C and glycerol-2-^{3}H, as previously described (7). For these studies a mixture of 2-^{3}H-glycerol triolein and glycerol-1, 3-dioleate-2-oleate-1-^{14}C in the ratio of approximately 10:1 provided a specific activity of 1.6 μc/mg for ^{3}H and 0.16 μc/mg for ^{14}C. Verification of the purity of these glycerides was carried out frequently over the course of many months by thin layer and gas-liquid chromatography and by enzymatic hydrolysis. The purity of the glycerides was within 4% of the indicated composition of each compound at the time of each study.

Experimental design. In each instance the test glyceride was administered after a 10-12 hr fast. Patients were studied in the Clinical Research Center of New York University School of Medicine at Bellevue Hospital. Five male patients were the subjects for this study. Thoracic duct cannulation was carried out in four of the patients for therapeutic or diagnostic reasons related to their primary disorder; one patient, W.H., was a volunteer.

B.B. was a 50 yr old Negro male with a history of rheumatoid arthritis for 3 yr before study. Thoracic duct cannulation was performed for study of rheumatoid factor in lymph. The test meal for this patient was 170 mg of a mixture of 1- and 2-isomers, in a ratio of 30:70, of doubly labeled monostearin made into micellar solution with 2 ml of Emulphor EL 620,[1] and 23 ml of carbon dioxide-free water at 40°C. 20 ml of the solution was instilled via Miller-Abbott tube into the duodenum. The tube flushed with warmed water and withdrawn. 2 hr later the patient was given 100 g of beef, and 200 ml of fruit juice; 3 hr later he began a regular diet and was encouraged to take fluids. Egg nogs were frequently given to maintain fluid and caloric balance; the marked variations in triglyceride content of the lymph samples reflect both fluid and fat content of the diet. Collections were continued for 48 hr.

J.K. was a 73 yr old white man with known hypertension for 3 yr. Admission to the hospital was prompted by abdominal pain. Exploratory laparotomy revealed a large retroperitoneal lymphosarcoma. It was planned to treat this tumor by retrograde instillation of streptonigrin into the thoracic duct. The monoglyceride study was carried out 2 days after cannulation and before any anti tumor therapy. The test meal for this patient was 180 mg of a mixture of 1- and 2-isomers, in a ratio of 80:20, of doubly labeled monostearin in a micellar solution; the monostearin was dissolved in 4 ml of ethyl ether to which 0.5 g of oleic acid dissolved in 3 ml of ethyl alcohol was added. Pure sodium taurocholate, 0.5 g, was dissolved in 20 ml of warmed phosphate-buffered saline solution (15 ml of 0.01 M NaH_2PO_4, 30 ml of 0.01 M Na_2HPO_4, and 40 ml of 0.45% NaCl). The bile salt phosphate buffer mixture was added to the warmed ether-alcohol fatty acid-monostearin mixture with continued mixing and heating until ether odor was no longer detectable. The initial pH was 6 and NaOH (1 N) was added until the pH was 7.2; the solution was initially cloudy but became water clear with formation of a stable micellar solution at pH 7.2. 20 ml of this solution was instilled into the duodenum and the tube was washed with 15 ml of additional warm water and withdrawn. Regular feedings were started 6 hr later. These also included egg nogs which markedly altered the flow and fat content of the lymph. The cannula became occluded 16 hr after feeding of the test meal, and no additional lymph was collected. Radioactivity was noted in the 4 and 6 hr samples.

[1] Emulphor EL 620, polyoxyethylene triricinoleate, General Aniline & Film Corporation, New York, N. Y.

W.H., was a 55 yr old white man admitted because of purpura and peripheral vascular disease. Extensive hematologic study failed to reveal any cause of the purpura. Thoracic duct cannulation was carried out for study of labeled triolein absorption. Duodenal cannulation was unsuccessful in this patient. 4 hr before the administration of the radioactive meal, 40 g of triolein and 100 ml of fruit juice were given by mouth. The labeled triolein was mixed with aspirated gastric contents and reinjected into the gastric tube. Additional warmed water was given and the tube withdrawn. Fluids were given 2 hr later and regular meals 6 hr later. Egg nogs were used as supplementary feedings. Collections via cannula were continued for 36 hr. Virtually no radioactivity was recovered in the 0–2 hr collection after feeding. Radioactivity was detected in plasma samples at 2 hr after the test meal.

E.G., a 59 yr old white man, was admitted because of a recurrent pulmonary infection associated with his chronic pulmonary disease. Thoracic duct cannulation was performed at the time of a supraclavicular lymph node biopsy; the thoracic duct lymph and lymph node were studied for acid fast infection, malignant granulomata and fungi. The labeled triolein study was carried out on the first afternoon after cannulation; the study of the absorption of labeled free oleic acid and unlabeled triolein was performed on the 3rd day after cannulation and 42 hr after the labeled triolein study. The patient was given 30 g of unlabeled triolein mixed with fruit juice. One-half hour later, intestinal contents were aspirated from a tube previously passed into the duodenum. About 7 ml of the intestinal aspirate was mixed with the doubly labeled triolein and 0.5 ml of unlabeled triolein, and a homogeneous emulsion was formed by sonication with a BioSonik Model II Sonicator (Bronwill Scientific, Division of Will Scientific, Inc., Rochester N. Y.) for 30 sec. This material was introduced into the tube and the tube flushed with tap water. The tube was withdrawn 30 min later. The patient received a normal diet 2.5 hr after the triolein; lymph samples were continually collected from the time of surgery, for 42 hr. Radioactivity was noted in the 6 hr sample. The labeled oleic acid unlabeled triolein study (Study V) was begun in this patient when radioactivity in lymph samples had fallen to a low level. 20 ml of unlabeled triolein and 50 μc of oleic acid-1-^{14}C (specific activity 24.0 mc/mmol) were mixed with tomato juice and taken orally by the patient. During the 16 hr before the test meal solid food had been withheld but fluids were freely given. The patient's usual diet was resumed 16 hr after the labeled oleic acid-unlabeled triolein mixture had been ingested.

S.P., a 36 yr old white man, was admitted because of chronic alcoholism for 16 yr, and ascites for 2 months. Diuresis was obtained after salt restriction, and abdominal paracentesis of 4 liter. Thoracic duct cannulation was carried out to assay pancreatic enzyme activity in thoracic duct lymph. The radioactive glycerol study was carried out on the 4th day after cannulation. Glycerol-^{3}C (40 μc) obtained from Nuclear-Chicago was mixed with 10 ml of warm water. A micellar solution was made as follows. Na taurocholate (0.84 g) and Na glycocholate (1.28 g) (National Biochemicals Corp., Cleveland, Ohio) were dissolved in phosphate buffer (0.077 M Na$_2$HPO$_4$ 0.105 M NaH$_2$PO$_4$). Monoolein (0.50 g), hexadecanoic acid (0.025 g), palmitic acid (0.06 g), and unlabeled glycerol (0.038 g) were dissolved in ethyl alcohol:ethyl ether mixture (3:5 v/v). The buffered bile salts were added to the ethereal-alcohol lipid solution with continuous stirring and heating to 45°C. The solution (total volume 150 ml) was clarified by the addition of NaOH to pH 7.1. The solution was administered by intraduodenal tube during a 45 min period; the 10 ml of radioactive glycerol solution was admixed with the micellar solution at 35 min after start of instillation, and the tube flushed with additional nonradioactive solution.

Analytic procedures. Lymph samples were collected into iced containers and stored at 4°C until analyzed. The samples were strained through gauze, the volumes recorded, and an appropriate fraction extracted with 20 volumes of chloroform:methanol 2:1 (v/v). Separation into two layers was achieved by either large volumes of acid water (0.05% H$_2$SO$_4$) or by smaller volumes of the dilute acid with centrifugation and washing of the chloroform fraction three times (8). Chloroform extracts were taken to dryness, and scintillant solution (2,5-diphenyloxazole, 4 g per liter of toluene, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene 100 mg per liter of toluene) was added. Samples were counted in a Tri-Carb (Packard) liquid scintillation spectrometer. Correction was made for isotope overlap in each channel; disintegrations per minute were calculated from efficiency data using known standards. Samples of the original meal were extracted in similar fashion and counted. Plasma samples were treated similarly. Quenching activity was approximately the same for all samples.

Triglyceride analysis was carried out on extracts of lymph by the method of Van Handel and Zilversmit (9); total lipids were estimated by the potassium dichromate method of Bragdon (10). Chylomicron fractions were prepared from lymph samples by centrifugation in a Spinco Model L ultracentrifuge at 26,000 g at 15°C either in a No. 40 rotor or in a No. 30 rotor. The creamy top layer was suspended in buffered phosphate-EDTA solutions and recentrifuged, as previously described by Kayden, Karmen, and Dimson (11). Lymph and chylomicron fractions were separated by thin layer chromatography on Silica Gel G (Brinkmann) plates in a solvent system of petroleum ether:ethyl ether:glacial acetic acid 70:30:0.8. Standards and unknowns were made visible by iodine vapor. Fractions of cholesterol ester, free fatty acids, glycerides, and phospholipids were scraped from the plate and either counted directly with scintillant solution, or extracted with chloroform and methanol, dried, and counted with scintillant solution in the liquid scintillation spectrometer.

Identification of the amount of labeled fatty acid present in the 1- and 3 positions in comparison with the 2 position was accomplished by enzymatic hydrolysis of the isolated triglyceride fraction, which was separated from other lipids (12) by silicic acid column chromatography.

Results

Study I, B.B., Table I. Total recovery of each isotope was 53% ^{3}H and 57% ^{14}C. Most (96%) of the total recovered ^{14}C-fatty acid was collected in the 8 hr period after feeding. During this period of absorption, the ratio of lymph isotopes $^{3}H/^{14}C$ to meal isotopes $^{3}H/^{14}C$ was initially 1.05, then 0.95, and then fell to 0.60 (Fig. 1). The fraction of fatty acid label in the 2 position was initially very similar to that of the test meal, but then decreased as absorption continued.

Study II, J. K., Table I. Total recovery of each isotope was 35% ^{3}H and 49% ^{14}C. The major portion (96%) of the total ^{14}C-fatty acid recovered was obtained during the first 8 hr after feeding. The isotope ratios in lymph triglycerides were similar to that of the test meal for 4 hr after feeding and then changed to 0.70 (Fig. 1). In this study the distribution of the fatty acids in the triglycerides was determined from the isolated chylomicron fractions, rather than from whole lymph samples. There was very little deviation in the per cent of ^{14}C-fatty acid at the 2-position in the lymph compared to the meal.

Study III, W.H., Table I. Total recovery of each isotope was 35% ^{3}H and 36% ^{14}C. Despite a large volume of lymph (360 ml), the samples for the first 2 hr after feeding had little radioactivity (< 0.1% ^{3}H and < 0.02% ^{14}C). During the subsequent 7 hr, there was collected in the lymph lipids 88% of the total carbon-labeled fatty acid that was recovered; the glycerol-to-fatty acid ratio in

TABLE I

Composition of thoracic duct lymph after gastric or duodenal instillation of doubly labeled glycerides

Time of meal	Lymph volume	Triglyceride	Lymph ^{14}C* / Total lymph ^{14}C × 100	$^{3}H/^{14}C$ ratio‡ / $^{3}H/^{14}C$ meal	% ^{14}C in 2-position of glyceride
hr	ml	g			
Study I (B.B.) 1-(2-monostearin, 30:70; total recovery ^{3}H 53% ^{14}C 57%					
0–2	155	0.44	29	1.05	70
2–4	170	0.90	45	0.95	60
4–6	195	1.61	16	0.81	42
6–8	245	5.19	6	0.69	34
8–14	520	11.8	4	0.60	20
14–20	565	13.9		0.58	20
Study II (J.K.) 1-(2-monostearin 80:20; total recovery ^{3}H 35% ^{14}C 49%					
0–2	85	0.26	34	0.98	20
2–4	145	0.73	41	1.03	18
4–8	195	12.4	31	0.70	18
8–12	420	0.61	4	0.42	13
12–16	40	0.69		0.46	16
Study III (W.H.) triolein, labeled fatty acid in 2-position; total recovery ^{3}H 35% ^{14}C 36%					
0–2	360	1.25	<1	1.12	>98
2–5	320	2.69	46	0.99	78
5–9	540	17.6	42	0.99	55
9–15	160	5.05	12	0.88	43
15–17	650	5.49		0.77	39
17–21	630	9.33		0.70	40
21–25	625			0.77	35
Study IV (E.G.) triolein, labeled fatty acid in 2-position; total recovery ^{3}H 24% ^{14}C 25%					
1–2	19	0.70	35	1.01	91
2–3	60	2.79	40	0.98	70
3–4	28	1.76	5	0.91	74
4–6	130	5.06	11	0.86	71
6–8	93	1.72	8	0.88	63
8–10	50	1.17		0.88	65
10–12	15	0.02		0.81	61
12–14	87			0.86	76

* Figures in this column are the percentage of recovered ^{14}C-fatty acid collected during each time interval.
‡ Glycerol-^{3}H label; ^{14}C-fatty acid label.

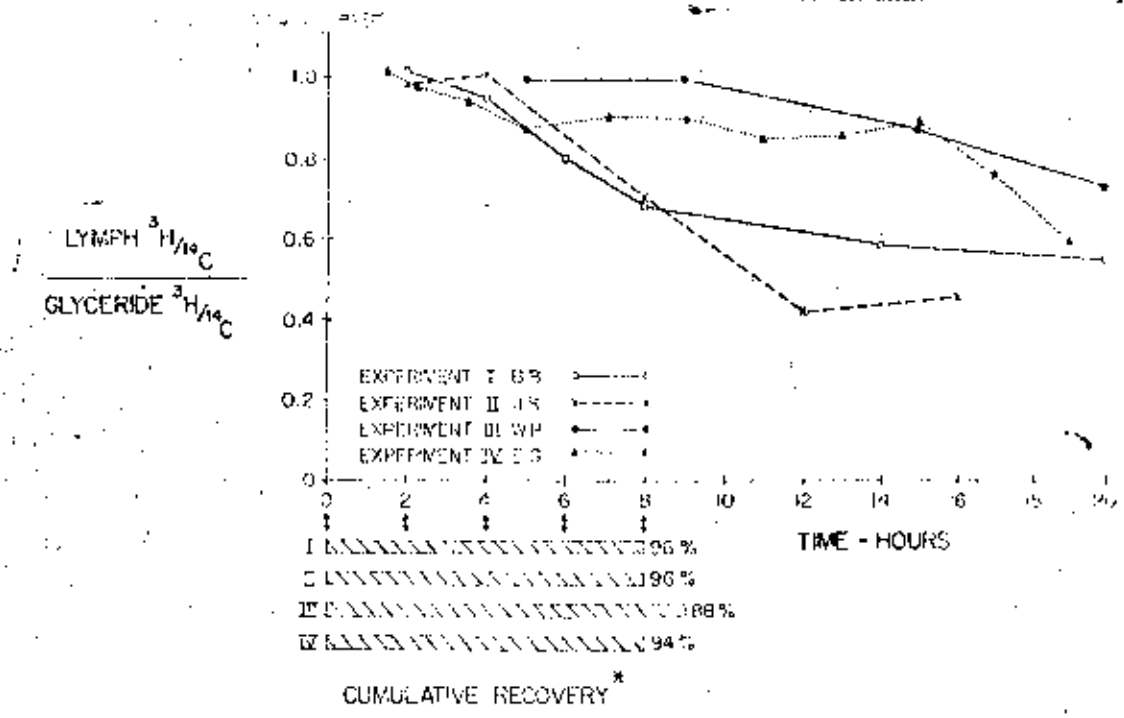

FIG. 1. COMPARISON OF ISOTOPE RATIOS IN HUMAN LYMPH LIPID AND DIETARY TRIGLYCERIDES AFTER GASTRIC OR DUODENAL ADMINISTRATION OF DOUBLY LABELED GLYCERIDE. * *Cumulative recovery* indicates the period during which the indicated per cent of the total labeled fatty acid that was recovered appeared in lymph lipids.

lymph lipids was the same as the isotope ratio of the meal (Fig. 1). 78% of the carbon label was in the 2-position for the first 3 hr after feeding and then fell for the subsequent collections.

Study IV, E.G., Table I. The total recovery of each isotope was 24% ^3H and 25% ^{14}C. The per cent of labeled fatty acid in the 2-position of the test triolein was 91. The 2nd hr sample, which contained 35% of the ^{14}C label that was recovered, had 70% of labeled fatty acid in the 2-position of the lymph triglyceride. This value for distribution of the labeled fatty acid was maintained for 4 hr, and then fell during the subsequent 8 hr.

Study V, E.G., Table II. The total recovery of the oleic acid-^{14}C was 48%. A tracer amount of labeled free oleic acid along with 20 ml of unla-

TABLE II

Composition of thoracic duct lymph after oral administration of labeled (^{14}C) oleic acid and unlabeled triolein

Study V (E.G.); total recovery 48% ^{14}C

Time	Lymph volume	Triglyceride	Lymph ^{14}C / Total lymph ^{14}C × 100	% ^{14}C in 2 position of glyceride
hr	ml	g		
0–1	48	0.25	<1	25
1–2	57	0.71	6	15
2–2.5	33	1.52	18	12
2.5–3	23	1.23	15	12
3–4	60	2.13	27	15
4–6	61	1.62	10	18
6–8	66		7	19
8–10	72		4	18
10–12	70			
12–16	130		12	22
16–20	110			
20–24	175			24

* Figures in this column are the percentage of recovered ^{14}C.

TABLE III

Absorption of radioactive glycerol into thoracic duct lymph after duodenal instillation

Study VI (S.P.) [^{14}C]-glycerol; total recovery ^{14}C 4.1%

Time	Lymph volume	Lymph ^{14}C Total lymph ^{14}C Σ=100
hr	*ml*	
0–0.5	20	0
0.5–1.0	15	3
1–2	130	55
2–3	80	30
3–5	155	9
5–18	360	3

* Figures in this column are the percentage of recovered ^{14}C.

beled triolein was administered to estimate the available hydroxyl sites for esterification on the absorbed monoglycerides within the intestinal cell. In this study, it is assumed that the labeled free oleic acid is almost exclusively utilized for esterification of the hydroxyl groups on the absorbed monoglycerides (from triolein), and not for synthesis via the glycerol phosphate pathway, and in addition, that there is no positional preference for oleic acid at the 1- and 3 positions of the monoglycerides. If the fatty acids were esterified to the absorbed monoglycerides in an entirely random distribution, 33% of labeled oleic acid should have been found at the 2-position of the lymph triglycerides. The data show that during the bulk of lipid absorption only 12 to 13% of labeled oleic acid was present at the 2-position, which indicates that considerably more hydrolysis and reesterification were taking place at the 1- and 3 positions than at the 2-position.

Study VI, S.P., Table III. In contrast to the greater recovery of glycerol when fed as a glyceride, only 4.1% of the radioactive glycerol was recovered from thoracic duct lymph lipid. Radioactivity appeared one-half hour after the administration of the material, and more than 88% of the total amount recovered appeared during the first 3 hr of lymph collection after instillation of the radioactive meal into the duodenum. Assay for radioactivity after fractionation by column chromatography and thin-layer chromatography revealed that the radioactivity was almost exclusively in the triglyceride fraction.

Discussion

The absorption of intact monoglycerides into the intestinal cell wall of rats was demonstrated in 1959 by Skipski, Morehouse, and Deuel (4) who used 1,3 dioleyl 2-deuterostearyl glyceride-^{14}C. Additional observations in the rat by Reiser, Bryson, Carr, and Kuiken (13) with doubly labeled monoglycerides as test meals had previously provided evidence that monoglycerides were absorbed intact into the intestinal cell wall and reesterified in the intestinal cells to triglycerides which then appeared in thoracic duct lymph. These observations were based primarily on analyses of the ratio of the two isotopes, tritium for glycerol and ^{14}C for fatty acids. Mattson and Volpenhein (5) extended these observations by analyzing in the rat the amount of fatty acid label at each position of lymph triglycerides, after feeding monoglycerides, free fatty acids, labeled fatty acids, and triglycerides synthesized with labeled fatty acids at various positions. From these studies they concluded that hydrolysis of the dietary triglycerides in the intestinal lumen yielded 72 parts 2-monoglycerides, 6 parts 1- and 3-monoglycerides, and 22 parts free glycerol. The 2-monoglycerides, approximately three-fourths of the dietary triglycerides, entered rat intestinal cells intact, and were reesterified to triglycerides without further alteration of the fatty acid at the 2-position.

The particular role that monoglycerides have in furthering human emulsification of dietary lipids has been presented by Hofmann and Borgström (14). Analysis of intestinal lumen contents of man after fat feeding has shown a separation of glycerides into two phases: an oil phase which contains mainly diglycerides and triglycerides and some fatty acid, and a micellar phase which is a combination of bile salts, free fatty acids, and monoglycerides. From the micellar solutions, monoglycerides and free fatty acids are absorbed through the intestinal wall, leaving the bile salts within the lumen to form additional micelles.

The fate of the free fatty acids within the mucosal cell is determined by their chain length and saturation; those of less than 12 carbons are preferentially absorbed by rats directly into the portal blood, whereas longer chain fatty acids are incorporated mainly into triglycerides, and appear in lymph chylomicrons (15). Monoglycerides within epithelial cells may be esterified to di- and triglycerides or cleaved to glycerol and free fatty acids. The 2-monoglycerides are esterified especially rapidly to triglycerides, but esterification

of 1-monoglycerides lags and these are more likely to undergo hydrolysis particularly if the fatty acids are of short chain length as shown in studies in hamsters (16).

The course of drainage from intestinal lymphatics into the thoracic duct has considerable anatomic variations and is not solely into the left thoracic duct. Although the size of the cannula introduced into the duct makes it unlikely that there is much leakage around the cannula into the venous system, there are other lymphatic-venous channels that are potentially functional and may even actively serve as pathway for intestinal lymph. It has not been possible to estimate the extent of these shunts by sampling plasma and measuring the concentration of radioactivity derived from the feeding, as the turnover time for various lipid classes and the size of the vascular pool make calculations for these channels almost impossible. But blood samples taken during the course of experiments II, III, and IV revealed radioactivity in the triglyceride fraction of plasma. Recoveries of labeled fatty acids in lymph triglycerides in man range from 25 to 60%; these values are less than the 60 to 90% recoverable in rat studies with labeled fat and cannulation of the main lymph trunk in the abdomen.

The data on the absorption of glycerides obtained from these four patients demonstrate that 2-monoglyceride and its accompanying free fatty acids are the main products of triglyceride digestion and are also the principal forms in which fat is absorbed in man, just as in other animal species. It was formerly held that complete hydrolysis of triglycerides to glycerol and free fatty acids with absorption of these digestion products was a pathway for fat absorption. The results obtained in our studies demonstrate that this pathway can be of only limited importance in man. In Study VI, glycerol-^{14}C was given into the duodenum in combination with micellar solution of bile salt, monoglyceride, and fatty acid. Such a mixture was shown by Saunders and Dawson in rats (17) to give maximal incorporation of glycerol into lymph triglycerides. It should be noted that only 2.5 g of lipid was given in the micellar solution along with the radioactive glycerol. This may have reduced the amount of labeled glycerol (4.1%) recovered from the thoracic duct lymph since the supply of fatty acids may have been limited at the site of glycerol absorption. The amount recovered here is greater than that reported by Holt, who recovered only 0.7 to 1.2% of the administered glycerol-^{14}C in the 24 hr urine collection of a patient with chyluria (18). The labeled glycerol in the thoracic duct lymph was almost exclusively present as triglyceride, as determined by thin-layer chromatography of the extracts of lymph lipids. The remainder of the glycerol was presumably absorbed directly into the portal blood.

Since this study in man shows that little of dietary free glycerol was incorporated into lymph lipids, then free glycerol resulting from the complete hydrolysis of triglycerides in the lumen of the intestine similarly would be incorporated only to a very limited extent into the lymph triglycerides. When labeled glycerol was fed as glycerol-glyceride, however, from 35 to 53% of the label was recovered in the lymph lipids, and absorption of glycerol therefore must have taken place as a glyceride. This is supported by the ratio of recovered ^{3}H (glycerol) and ^{14}C (fatty acid). In all four studies, during the period when most of the experimental lipid was being recovered, the ratio of the two labeled moieties in the lymph was approximately that in the diet (Fig. 1). Therefore, during the processes of digestion, absorption, and resynthesis, the glycerol and fatty acids remain associated.

The distribution of the labeled fatty acid on the triglycerides of the lymph lipids in Studies I, III, and IV shows that the main bulk of these lipids was absorbed without hydrolysis of the fatty acid located at the 2-position of the dietary glyceride. In these three studies more than half of the labeled fatty acid was still in the 2-position by the time it had reached the lymph. Only in the later time periods, after the main bulk of the dietary lipids had been absorbed, did these values drop.

Study V, in which labeled free oleic acid was given with a large amount of unlabeled tristearin, supports the concept that greater hydrolysis and resterification must take place at the 1- and 3-positions of the dietary fat, since the presence of label that could be introduced into the 2 position was only 12–13%, and not 33%, if random labeling occurred.

In Study II, when a mixture of 1- and 2-monostearin in ratio of 80:20 was fed, about 18% of the labeled fatty acid was in the 2 position of the lymph

triglyceride, a value similar to that in the dietary fat. In this patient the total recovery of ^{3}H (glycerol) of 35%, although less than that of the ^{14}C (fatty acid) of 49%, indicates that 1-monoglyceride was also absorbed intact. The ratio of the two isotopes in lymph lipids initially was not different from that of the dietary fat. The fate of absorbed 1-monoglycerides in the intestinal wall has been studied in animal experiments; both 1- and 2-monoglycerides are subject to hydrolysis by the intracellular monoglyceride lipase (glycerol monoester hydrolase) (19). However, 2-monoglycerides are more rapidly esterified to triglycerides (10) and incorporated into chylomicrons for delivery into the intestinal lymphatics. Under the conditions of this study, it is likely that 1-monoglycerides were also absorbed intact and esterified.

The observations reported here, together with studies on the hydrolysis of triglyceride in man, and supported by the more extensive absorption studies in animals, suggest that in man after fat ingestion, complete hydrolysis to glycerol and free fatty acid occurs to only a limited extent, especially in the first hours after a meal during which most of fat absorption occurs. The data from these studies indicate that the main route of human fat absorption into the lymph is by the direct esterification of 2-monoglycerides, derived from triglycerides, by the free fatty acids of the intestinal lumen that arise as products of pancreatic lipolysis, or by free fatty acids within the intestinal cell that are derived from monoglyceride hydrolysis and possibly from the circulating free fatty acid pool (11). Glycerol-3-phosphate derived from glucose metabolism or from phosphorylation of free glycerol can be esterified to phosphatidic acid, which then forms diglycerides (20). If large amounts of free fatty acids are present in the diet without glycerides, the glycerol portion of the lymph triglycerides would be formed from glycerol phosphate, with esterification of absorbed free fatty acids via phosphatidic acid to di- and triglycerides (21).

Calculations on the mode of triglyceride absorption in the extensive observations in animals indicate that as much as 72% of the triglyceride glycerol is absorbed as the 2-monoglyceride and reesterified to triglyceride. Our studies suggest that in man also, on the order of three-fourths of the dietary triglycerides may be absorbed as 2-monoglyceride and incorporated intact into lymph triglycerides. The monoglyceride pathway of fat absorption appears, therefore, to be the predominant route for the normal absorption of dietary triglyceride.

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Physiological Properties of Blown Oils

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By blowing oils rich in polyenic acid at higher temperatures preparations are obtained the toxicity of which increases initially simultaneously with increased blowing intensity; the toxicity reaches a maximum and subsequently falls off again. The toxicity of said blown oils can presumably be attributed to oxypolymers. The limiting (critical) dose which can likewise be expected to have the toxic effect of such blown oils, was determined in the rat to the amount of approximately 0.45 to 0.90 g per kg of body weight.

Substantial literature is available by now concerning the physiological effects of blown oils. We are indebted to F. CUSTOT[1] for a survey of studies published up to 1959. Further important findings were published later[2-7]. These works make it appear probable that the oxypolymers, specifically the fraction of the dimers, are to be held principally responsible for the toxicity of certain blown oils. Since only very low peroxide numbers are found for oils blown at higher temperatures, the fatty acid-peroxides are practically excluded as cause of the different biological effects. But they are obviously responsible for the toxic effects of oils blown at low temperatures. According to submitted investigations, however, no different bilological properties were found with numerical peroxide factors below 100[8,9]. Fatty acid-peroxides have a strongly irritating effect. Therefore considerably inflammation-caused changes are found in the stomach-intestinal area in case of fats with high peroxide numbers. Such inflammatory reactions do not occur with the toxic effects of oils blown at higher temperatures, which consititues another indication to the effect that their toxicity is not caused by peroxides.

Data in literature concerning the result of feeding tests with blown oils show considerable differences. They range from tolerance, pratically without symptoms, to more or less serious growth inhibitions, to high toxicity, i.e. to a high mortality rate of the animals within a brief period of time. These discrepancies are mainly due to the fact that systematic investigations concerning the correlation between effect and treatment method are almost completely lacking. Most of the reported findings are related to specific preparations, i.e. they are, to some extent, merely "snapshots" from a process which takes place during a prolonged period of time. This prompted us to undertake such systematic investigations concerning the physiological effects as a function of the treatment intensity.

Furthermore, we were interested in the mechanism of the toxic effect, on which almost no data was available. We therefore attempted to gain insight into the mode of action of blown oils by means of histological, biochemical and physiological investigations

Material and Methods.

Feeding tests 1 and 2.

The characteristic factors of the basic soybean oils and of the blown oils as well as the blowing conditions are compiled on Table 1. The blowing temperature amounted in each case to 180°C. The blowing took place for the oils Sj 1 to Sj 4 in charges of 3 kg each; for oils Sj 5 to Sj 8, in charges of 8 kg.

The determination of the characteristic factors was carried out with the following methods: iodine number according to H.P. KAUFMANN[10], peroxide number according to C.H. LEA[11], epoxy number according to L. KROLL[12], hydroxy number according to the DGF-unit method[13], and acid number according to the DGF-unit method[13].

Feeding test 1 involved the investigation of growth, feed intake, protein efficiency and water intake for the oils Sj 1 as control, and for the blown oils Sj 1 to Sj 4; groups of 20 male rats each were used; the rats had an initial weight of 50 to 60 g (Sprague-Dawley breed; breeder: GASSNER/Munich). The duration of the test was 5 weeks. The rats were kept in single cages with wire floor. The temperature during all feeding tests amounted to 22 ± 1°C, and the realtive air humidity was 50 to 60%. The determination of

the feed-and water intake took place as follows: a specific quantity was weighed and the collected remnants per animal were weighed back each week. The animals were weighed once per week. The diet was composed as follows: 20% fat, 51 % mondamin, 20% casein, 5% salt mixture according to SHAW, 2% cellulose and 2 % yeast.

The following vitamin doses were supplied: per animal and per day, 100 γ thiamine, 100 riboflavine, 1 mg niacine, 100 calciumpantothena 50 γ pyrodoxine and 15 mg cholinchloride; in addition, each animal received per week 30 IE vitamin A-palmitate and 3 mg tocopheryl-acetate in 0.1 ml arachis oil.

Table 1.

Characteristic factors of the blown soybean oils used during the Feeding Tests 1 and 2.

	Sj K	Sj 1	Sj 2	Sj 3	Sj 4	Sj KW	Sj 5	Sj 6	Sj 7	Sj 8
Blasezeiten Std. 1)	—	5	10	15	20	—	2.07	3.0	4.8	7.6
Luftmenge m³/kg 2)	—	0.063	0.192	0.104	0.190	—	0.7	1.1	1.7	5.0
Jodzahl 3)	125	121	115	100	109	130	110	96	75	70
Peroxydzahl 4)	3.2	2.0	2.4	1.8	3.7	13.3	14.2	12.1	7.6	7.4
Säurezahl 5)	0.25	0.41	0.55	0.58	0.80	0.0	1.6	2.4	2.8	3.7
Epoxydzahl 6)	0.1	1.30	1.90	1.44	2.60	0.27	6.5	8.1	8.1	8.4
Hydroxylzahl 7)	3.2	2.8	4.4	4.7	8.6	0.0	20.7	27.6	33.0	38.5
Verseifungszahl 8)	192	193	196	195	198	184	196	199	197	208

1) Blowing periods, hours
2) Air volume, m³/kg
3) Iodine number
4) Peroxide number
5) Acid number
6) Epoxide number
7) Hydroxyl number
8) Saponification (hydrolysis) number

During Feeding Test 2 which had a duration of 8 weeks, the oils Sj KW and the blown oils Sj 5 to Sj 8 were tested with the same method. Each group included 20 males and 20 females of the same fat breed as used for Feeding Test 1. The fat resorption was additionally investigated here. The diet was composed as follows: 20% fat, 33.3% skim milk powder, 30 % mondamin, 10% casein, 2% cellulose, 2% yeast and 0,7% salt mixture.

Vitamins were added as for Feeding Test 1.

Feeding Test 3.

The blown soybean oil *) used for this test had the following characteristic factors: iodine number 109, peroxide number 8.6, hydroxyl number 20,5, epoxide number 0, viscosity (at 20°C) 141 cp.

Feeding Test 3 was likewise implemented with Sprague-Dawley rats. Keeping- and feeding technique corresponded to (methods used for) Feeding Tests 1 and 2. The diets were composed as follows(%):

	I	II	III
Blown soybean oil	20	5	2.5
Olive oil	-	15	17.5
Mondamin	38	38	38
Skim milk powder	39.5	39.5	39.5
Cellulose	2	2	2
Salt mixture	0.5	0.5	0.5

During Tests I and II the fodder was supplemented with 1/10 of a vitamin B-complex pill (HOFFMANN-LA ROCHE) and 2 drops of codliver oil per animal and per day. During Test III the vitamin B-complex was substituted by 1 drop of Protovit (liquid) of HOFFMANN-LA ROCHE. The topopherol-doses are shown on Table 4.

Each group included 20 males and 20 females. The duration of the Tests I and II was 12 weeks, and 16 weeks for Test III.

Determination of the fat resorbtion.

a) Utilization: For this purpose, the rats were kept in metabolism cages. The feces excreted during one week were dried in the vacuum desiccator over silicagel and the dry weight was determined. 1.0 g dry feces was weighed into cut glass test tubes, mixed with 5 ml of nHCl and heated for 10 minutes in a water bath. The feces was then was then finely suspended in the hydrochloric acid with the glass stamper and added after cooling to a mixture of 5 ml petro-ether and ether, 1 : 1 .

*) We wish to thank Prof. Dr. h.c. H.P. KAUFMANN for providing the oil.

The cut glass containers were sealed after waterproofing of the cuts with glycerine and vigorously agitated overnight. Centrifugation was carried out on the following day; the petro-ether-ether layer was decanted into a small glass vessel for weighing and the extraction was repeated with the same method with an additional 5 ml of the solvent-mixture. The united extracts lost most of the solvents while standing overnight. The rest was removed through evacuation in the vacuum desiccator over silicagel. The extracted fat was subsequently weighed. The consumed fat quantity was known on the basis of the food analysis and the determination of the feed intake (see above). The utilization ("apparent fat reabsorption") will then result as:

$$\text{Utilization} = 100 \left(1 - \frac{\text{fat excretion}}{\text{fat intake}}\right.$$

The rats of Feeding Test 2 served for the determination of the utilization.

b) Duration of stay in stomach and intestine:

Rats weighing 185 to 235 g were used for these tests. First, the animals received the standard rat feed of LUTZ and water ad libitum; they were kept at an ambient temperature of $22^{o} \pm 2^{o}C$. 48 hours before the start of the test feed was withheld, while drinking water remained available for them. Then each animal received 200 g of oil per each 100 cm^2 of body surface through the esophagus tube. The precise determination of the oil volume was carried out by weighing the used injector before and after the oil application. The animals were killed 3 and 6 hours after the oil application. Following the fastening of ligatures to the cardia, the pylorus, behind the passage to the colon and at the rectum, the stomach and the emerging intestine sectors were removed and the content was in each case rinsed with ether. After adding Na_2SO_4 for desiccation purposes, the extracts were left standing overnight. After additional rinsing with ether, they were then filtered into small glass tubes for weighing. The ether was evaporated and the residue weighed. The same blown soybean oil [illegible] was used for these tests.

c) Evacuation of fat through the intestinal lymph: Rats (SPRAGUE-Dawley) weighing 250 to 300 g were used for the tests. First, the animals received the standard rat feed of LATZ for some time, plus drinking water ad libitum; they were kept at a temperature of $22^{0}\pm2^{0}$C. 14 hours before the start of the test, feed was withheld. Under ether narcosis the abdominal cavity was opened, the intestinal lym duct is located and freed after careful preparation--as described by J.L. BOLLMANN and collaborators[14]-- and a fine PVC-catherther was inserted. After closing of the abdominal cavity the animals were placed into BOLLMANN-cages and the draining lymph was collected in graded measuring cylinders. The tests itself was actually started 1 hours after the termination of the operation, when the animals had recovered from the narcosis and constant drainage of lymph occurred. The animals then received 0.25 ml untreated or blown (010) soybean oil respectively, per 100 g of body weight, administered with the aid of the esophagus tube. After 1,2,3,4,5,6 and 24 hours the excreted lymph fluid was removed and the fat volume contained in the same was determined. The fat was extracted from the lymph according to BLOOR, the solvent was evaporated, the residue integrated with chloroform and dried over Na_2SO_4. The extract was subsequently desiccated in pre-weighed small glass tubes which were weighed after constant weight was reached.

Effect on the ester system of the stomach-intestine tract.

40 mice weighing [illegible] g each were used for the tests. Before the tests the animals received [illegible] dry feed and water ad libitum. 48 hours before the start of the test the dry feed was [illegible] and the animals received a [illegible] glucose-solution and tap water ad libitum. Then untreated and blown soybean [illegible] was [illegible] the animals through the esophagus tube in quantities of [illegible] 100 g of body weight, whereby a group of [illegible] animals was [illegible] each dosage. 2 hours after the application of the [illegible] g of body weight of an ink solution was administered [illegible] animals; the solution was made [illegible] part [illegible] water. 4[illegible] minutes after the application of the [illegible] were killed and the intestine as well as the [illegible] The ink conveyance was then verified in the [illegible] from the pylorus and the ink transport was expressed in percentage according to the length of the intestine.

Effect on the gall secretion

The test were carried out with rats weighing from 150 to 210 g. Prior to the test they received the LATZ standard rat feed and drinking water ad libitum. 16 hours before the start of the test the food was withheld, but drinking water remained available for them. On the test day the abdominal walls were opened under ether narcosis, a thin PVC-cannula was tied into the ductus choledochus, and a somewhat thicker PVC-cannula was ligated in the upper small intestine beneath the papilla vateri. After closing of the abdominal walls the animals were placed into BOLLMANN-cages and the draining gall was collected in graded measuring cylinders. 30 minutes after the termination of the operation the cholerese -tests were started. First, the gall secretion (void period) was verified. Subsequently 5 animals received 0,2 ml untreated soybean oil per 100 g of body weight through the small intestine cannula and 5 animals received the blown soybean oil 101. The gall secretion was observed up to 5 hours after the administration of the oil. For the purpose of balancing the fluid loss caused by the gall secretion, the animals received in intervals of 2 hours each adequante quantities of a physiological saline solution, applied intraduodenally. The gall volumes secreted by each animal were in each case correlated to the void period and expressed in percentages of said value.

Effect on the secretion of water, sodium and chloride by the kidney.

Rats weighing from 140 to 180 g were used for the tests. First, the animals received for some time the LATZ standard rat feed. 14 hours before the start of the test feed and water were withheld and 5 ml of a 2% NaCl-solution per 100 g of body weight was administered to them through the esophagus probe. On the following day they received through the esophagus probe 5 ml of tap water per 100 g of body weight, either separately (control) or simultaneously with the i.p. injection of 0,5 ml of soybean oil per 100 g of body weight (untreated oil, and the blown oil 010 respectively). 10 animals were used for each test series. In each case, two animals were placed into a metabolism cage.

The secreted urine volumes were collected for 1,2,3,6 and 24 hours after the start of the test. After the determination of the quantity the sodium was flame-photometrically determined and the chlorine was determined according to VOLLHARDT. The obtained values were uniformly converted to 100 g body weight.

Liver function test.

The bromosulfthalein-test was performed as follows: first, a blood sample was taken from the tail end which (sample) served as void value (Leerwert) for the compensation of the serum application color. After scabs had formed on the tail lesion, a bromosulfthalein volume of 25 mg/kg of body weight was injected into the tail vein and an isotonic citrate buffer solution spraying followed immediately, so as to eliminate dye residues from the cannula and to prevent their overflowing into the tail tissue. A stop watch was started immediately after the termination of the injection; 1 minute and 20 minutes after the termination of the injection one further blood sample each was taken from the tail end. The blood samples were drawn into capillary tubes having a diameter of 1,8 mm and a length of 110 mm. The capillary tubes were subsequantly unilaterally scaled by melting and centrifuged for 10 minutes at approximately 3000 UpM (revolutions per minute). The serum obtained thereby was transferred into a 20 mm^3-volume pipette and emptied into small test tubes which contained 0.48 ml n/10 NaOH. A 25-fold liquefaction of the serum is thus obtained; it shows a reddish-violet color, corresponding to its bromosulfthalein-content. The bromosulfthalein concentration was colorimetrically measured. The measurement took place with the EPPENDORF photometer at a wave length of 578 mμ, using semi-micro cuvettes. In each case, the serum sample taken before the dye injection served as void value (Leerwert).

The bromosulfthalein-retention in the serum, present 20 minutes after the dye injection, was expressed in % of the 1-minute value and computed according to the following equation:

$$R\ \% = \frac{E_{20}}{E_1} \cdot \frac{100}{}$$

R %= dye retention in %
E_{20}= extinction coefficient of the 20-minute value.
E_1= Extinction coefficient of the 1-minute value.

Effect on the dextran paw-edema of the rat.

Peanut oil was used for these tests which had been blown through for 5 hours at 150°C by an air jet of 1.5 l per kg and per minute.

18 rats weighing 150 to 200 g were subjected to the tests. The animals were divided into 3 groups of 6 animals each. Group 1 (control) received 0.5 ml water, Group 2 received 0.5 ml untreated peanut oil and Group 3 was given 0.5 ml blown peanut oil per 100 g of body weight through the esophagus probe. Immediately afterwards 0.1 ml of a dextran solution (Marcodex,10%, business firm AXOLL) was subcutaneously injected into one hind paw and the developing paw edema was determined according to the plethysmographic method of A. ENDERS and collaborators. The paw swelling was in each case expressed in percentages of the original datum and the timely development was graphically represented after the start of the test.

Effect on the enzyme system.

The following were used as enzyme systems:

a) 10% rat liver homogenate in 0.15% KCL-solution.

b) Cyclophorase-system from rat liver (according to D.E. GREEN and collaborators[16]).

c) Rat liver mitochondrion, prepared in 0.25 ml cane sugar solution according to W.C. SCHNEIDER[17].

The mixtures were agitated in WARBURG-containers at 37°C and the oxygen absorption was manometrically measured during 1 hour.

The starting solutions with liver homogenate contained:
0.5 ml homogenate with 4 to 5 mg N; 5 mol (gram molecules) substratum; 0.067 m phosphate buffer pH 7.4, with a total volume of 3 ml.

The starting solutions with cyclophorase-system contained:
0.5 ml cyclophorase-system, corresponding to 0.5 g fresh liver, 3 to 6 mg N per mixture; 50 mol substractum; 0.013 m phosphate buffer pH 7.4; 0.001 m ATP; 0.0012 m $MgSO_4$; 0.03 mol cytochrome c ; final volume: 3 ml.

The starting solutions with mitochondrion contained:
0.5 ml mitochondrion-suspension in 0.25 ml cane sugar solution, corresponding to 0.5 g fresh liver; 1 to 2 mg N per mixture; 50 µ mol substratum; 0.015 m KH_2PO_4; 0.029 m ATP and 0.015 µmol cytochrome c in a final volume of 2 ml.

TABLE 2

Weight increase, feed intake and protein efficiency during the feeding of slightly blown soybean oils.

	Sj K (Kontrolle)	Sj 1	Sj 2	Sj 3	Sj 4
Gewichtszunahme					
in 2 Wochen	45	44	42	40	35
in 4 "	105	98	99	96	88
in 5 "	127	127	126	118	110
Futterverzehr in g					
in 2 Wochen	107	117	107	112	98
in 4 "	248	265	248	260	234
in 5 "	326	350	326	340	300
Protein-Efficiency					
in 2 Wochen	2.37	2.21	2.35	2.11	2.09
in 4 "	2.45	2.19	2.36	2.18	2.27
in 5 "	2.31	2.14	2.29	2.06	2.10

Die Zahlen sind Mittelwerte von je 20 Männchen;
Fettdosis: 20 Gew.-% im Futter

1) (Control)
2) Weight increase
3), 5), 7) in . . . weeks. . .
4) Feed consumption in g
6) Protein efficiency
8) The figures are average values for 20 males each; fat dose: 20 weight % in the feed.

Determination of the organ weights and histological examination

The animals were exsanguinated during ether narcosis and subsequently dissected.

Statistical evaluation

The statistical evaluation of all tests took place according to the t-test of STUDENT[18].

Results

Feeding tests

The weight increases observed during Feeding Tests 1,2 and 3 as well as the feed consumption and protein efficiency are shown on Tables 2 to 4. The statistic evaluation of the tests with slightly blown soybean oils Sj 1 to Sj 4 (Table 2) are significant ($p = 0.001$) only for the weight increase difference between the control group and Group Sj 4. No detrimental effect resulting from the feeding of the less strongly blown oils Sj 1 to Sj 3 could be detected, even with high dosages; the feeding test, however, was of short duration only.

At variance from the above, the stronger-blown oils Sj 5 to Sj 8 proved to be toxic. As shown on Table 3,a considerable growth inhibition resulted in Group Sj 5, i.e. in 61% of the males and 57% of the Females, related in each case to the 6-week value. But no deaths occurred in this group. The growth delay was even more pronounced in Group 6 (67% for the males, and 69% for the Females). The highest toxicity rate became evident also when 3 animals of this group died (males only). The toxicity of the oil Sj 7 was even stronger. Growth delay in males amounted to 73% and to 61% in Females. A total of 14 animals (35% of the used 40 animals) died, whereby the mortality rate and growth inhibition was once more higher among males than among females. The still stronger-blown oil Sj 8 caused a similar growth inhibition, but the mortality rate was lower (7 rats, males only, corresponding to 18% of all animals used). The observed growth delays were highly significant with p 0.001, as compared to the control group Sj KW. The fact that the consumed feed volume remained unchanged and that the protein efficiency decreased considerably prove that the growth inhibitions were not due to a reduced feed consumption--possibly for organoleptic reasons; this indicates a serious disorder of the albumen metabolism.

Summarizing, it can accordingly be established that with increasing (oil) blowing intensity, the toxicity does at first increase, reaching a maximum with oil Sj 7; subsequently, the toxicity drops again. No strict correlation results hereby,however,with the characteristic oil data shown on Table 1 . In our opinion, the cause of the subsequent toxicit decrease can be ascribed to the deteriorating resorption of the fraction which is primarily held responsible for the toxicity. According to the data in literature and according to some of our own preliminary tests it is primarily the fraction of the dimers which shows a high toxicity rate.

TABLE 3.

Weight increase, feed intake and protein efficiency during the feeding of strongly blown soybean oils.

	1 Sj KW (Kontrolle)	Sj 5	Sj 6	Sj 7	Sj 8
2. Männchen					
3. Gewichtszunahme in g					
in 2 Wochen 4	49	16	12	12	14
in 4 „	117	45	36	31	40
in 6 „	168	66	54		
in 8 „	194	80	62 *	57	43
Zahl der Todesfälle	0	0	5	10	7
5 Futteraufnahme in g					
in 2 Wochen 4	120	105	91	92	109
in 4 „	278	229	206	192	213
in 6 „	462	381	331	334	356
in 8 „	646	522	401 *	444	464
6 Protein-Efficiency					
in 2 Wochen 4	2.19	0.76	0.66	0.65	0.69
in 4 „	1.90	0.89	0.80	0.72	0.85
in 6 „	1.64	0.78	0.75	0.64	0.72
in 8 „	1.36	0.70	0.70 *	0.58	0.47
7 Weibchen					
8 Gewichtszunahme in g					
in 2 Wochen 4	48	10	8	9	10
in 4 „	99	38	25	35	32
in 6 „	151	56	41	52	51
in 8 „	150	66	44 *	56	42
9 Zahl der Todesfälle	0	0	0	4	3
10 Futteraufnahme in g					
in 2 Wochen 4	120	96	95	87	102
in 4 „	275	216	205	191	202
in 6 „	442	352	325	316	344
in 8 „	620	484	388 *	481	439
11 Protein-Efficiency					
in 2 Wochen 4	2.17	0.56	0.44	0.55	0.51
in 4 „	1.77	0.80	0.56	0.82	0.72
in 6 „	1.34	0.72	0.58	0.74	0.67
in 8 „	1.10	0.62	0.52	0.58	0.42

12 * Bei der Gruppe Sj 6 betrug die Versuchsdauer nur 7 Wochen. Mittelwerte aus jeweils 20 Männchen und 20 Weibchen

1) (Control)
2) Males
3) weight increase in g
4) in . . . weeks . .
5) Feed intake in g
6) Protein efficiency
7) Females
8) weight increase in g
9) Number of deaths
10) Feed intake in g
11) Protein efficiency
12) *) For Group Sj 6 the test period duration was 7 weeks only. Average values of 20 males and 20 females in each case.

TABLE 4

Growth and protein efficiency during feeding of the blown soybean oil (k)

	1 20 % Öl in Futter			1 5 % Öl in Futter			1 2.5 % Öl in Futter		
	2 Kontrolle	3 geblasenes Sojaöl	geblasenes Sojaöl 4 + Tocopherol (2 mg/Woche)	2 Kontrolle	3 geblasenes Sojaöl	geblasenes Sojaöl 6 + Tocopherol (2 mg/Woche)	2 Kontrolle	3 geblasenes Sojaöl	geblasenes Sojaöl 4 + Tocopherol (2 mg/Woche)
Männchen (je 20) 5									
Gewichtszunahme in g 6									
in 2 Wochen 7	38 ± 1.2	22 ± 1.5	21 ± 1.3	40 ± 0.8	37 ± 1.4	34 ± 1.3	39 ± 1.6	39 ± 2.4	38 ± 2.0
in 4 „	76 ± 3.5	41 ± 2.0	38 ± 1.9	76 ± 1.2	62 ± 2.8	68 ± 2.4	77 ± 2.8	68 ± 2.5	71 ± 3.3
in 6 „	104 ± 2.4	—	67 ± 4.0	115 ± 3.7	98 ± 2.4	106 ± 2.6	128 ± 5.3	111 ± 3.9	110 ± 5.1
in 8 „	125 ± 3.3	—	92 ± 4.7	164 ± 3.6	127 ± 3.8	136 ± 3.1	151 ± 0.4	136 ± 5.2	142 ± 6.6
in 12 „							218 ± 5.1	194	197
in 16 „				192	186	192	241 ± 4.1	228	229
Gestorben in 8 Wochen 8	0	19	0	0	0	0	0	1	0
Protein-Efficiency 9									
in 4 Wochen 7	2.78	1.70	1.70	2.34	1.98	2.02	2.39	2.18	2.18
in 6 „	2.33	—	1.83	2.10	1.95	1.90	2.36	2.25	2.16
in 8 „	2.03	—	1.76	2.05	1.80	1.70	1.90	1.92	1.95
Weibchen (je 20) 10									
Gewichtszunahme in g 11									
in 2 Wochen 7	34 ± 1.3	20 ± 1.2	21 ± 1.1	39	33	31	36	38	37
in 4 „	69 ± 3.2	37 ± 3.2	39 ± 1.6	71	48	60	65	70	64
in 6 „	90 ± 3.2	—	64 ± 2.8	97	84	90	89	95	90
in 8 „	105 ± 2.9	—	82 ± 3.5	123	100	108	102	111	100
Gestorben in 8 Wochen 12	0	11	0	0	0	0	0	0	0
Protein-Efficiency 13									
in 4 Wochen 7	2.51	1.53	1.65	2.20	1.52	1.86	2.02	2.26	1.97
in 6 „	2.05	—	1.69	1.81	1.76	1.69	1.78	2.03	1.79
in 8 „	1.76	—	1.48	1.61	1.45	1.46	1.47	1.70	1.47

1) . . . % oil in feed
2) Control
3) blown soybean oil
4) blown soybean oil + tocopherol (2 mg/week)
5) Males (20 each)
6) weight increase in g
7) in . . . weeks
8) Died within 8 weeks
9) Protein efficiency
10) Females (20 each)
11) Weight increase in g
12) Died within 8 weeks
13) Protein efficiency

TABLE 5

Water intake during the feeding of blown soybean oil.

	Sj KW			Sj V			Sj VI			Sj VII			Sj VIII		
1	absolut [g]	g/g KG 2	g/g Futter	absolut [g]	g/g KW 2	g/g Futter	absolut [g]	g/g KG 2	g/g Futter	absolut [g]	g/g KG 2	g/g Futter	absolut [g]	g/g KG 2	g/g Futter
Männchen 3															
in 2 Wochen 4	240	2.35	2.00	249	5.29	2.57	245	5.71	2.74	216	5.48	2.35	242	5.61	2.22
in 4 "	557	3.27	2.00	557	5.30	2.48	596	5.88	2.60	457	5.52	2.35	486	5.02	2.29
in 6 "	925	4.18	2.00	896	7.11	2.30	857	7.12	2.59	687	7.01	2.06	727	6.49	2.04
in 8 "	1269	5.15	1.95	1211	8.58	2.82	—	—	—	1020	9.11	2.29	1066	10.10	2.29
Weibchen 5															
in 2 Wochen 6	253	2.91	1.94	245	3.67	2.34	228	4.05	2.30	253	3.70	2.53	256	4.15	2.28
in 4 "	514	3.36	1.87	522	6.29	2.42	517	6.54	2.52	514	6.00	2.68	561	6.52	2.78
in 6 "	828	4.48	1.87	862	7.84	2.45	847	8.92	2.61	828	7.67	2.49	885	8.53	2.58
in 8 "	1184	5.80	1.91	1190	9.91	2.46	—	—	—	1184	10.58	2.67	1218	12.58	2.65

1) Absolute
2) Feed
3) Males
4) in . . . weeks
5) Females
6) in . . . weeks

The growth tests 1 and 2 were performed with high dosages of 20 weight-% fat in the feed. We therefore investigated the limiting (critical) dose during an additional test (Feeding test 3): starting from said dose, harmful effects can be expected. At the same time, it was of interest for us to obtain knowledge regarding the effect tocopherol on the toxicity of the blown oil. Table 4 shows the result of this test. It is evident that while additions of tocopherol have a certain effect--they forestall high mortality, for example, in high (20%) oil dosages--they are not able to influence the considerable growth inhibition of 46% in males as well as in females (on the basis of the 4-week value). A growth inhibition of 23% in males which usually show stronger reactions during such tests than females, could still be verified when the blown oil content of the feed was 5%. However, a statistically certain growth inhibition could no longer be established when supplying feed with a 2.5% oil content. One animal of this group nevethless died; therefore the tolerability without symptoms of said oil can be assumed only for a dosage of less than 2.5%.

The water consumption of the animals was likewise detrmined in the course of Feeding Test 2. As known, increased consumption of water due to a malfunction of the water system is an early symptom of essential fatty acid deficiency. Proportionately to body weight, the water consumption of the animals in Group Sj 8 (Table 5)has definitely increased. An increased water consumption resulted for these groups as compared to the control (Sj KW) also relatively to the consumed feed volume. Furthermore, Table 5 shows that the modifications of the water system approximately paralleled the toxicity. It will therefore be necessary to discuss later whether the toxicity of blown oils might be attributed (entirely or partially) to a deficiency of essential fatty acids. At this time, we are still analysing the effect of modified fat feeding on the water consumption.

Utilization.

As Table 6 indicates, we found an utilization of 97 to 99% in control animals and in animals prior to the test; this coincides with data in literature concerning the utilization of the customary edible fats and oils. When feeding the strongly blown soybean oils Sj 5 to Sj 8, the utilization deteriorates. But the deterioration remains within moderate limits, and(substantially)does not exceed 10%. The single values of the animals were considerably scattered during said tests. It became evident,on the whole,that the utilization continued to deteriorate simultaneously with the increase of the oxidation rate. The minimum occurred in the group which had been given the oil Si 7. The still stronger-blown oil Sj 8 showed an utilization (rate) which was approximately the same as for Sj 7.

TABLE 6

Utilization of blown soybean oils (in %)

1 Gruppe		2 Leerwert	3 2 Wochen	3 4 Wochen	3 6 Wochen	3 8 Wochen
Sj KW	Männchen 4	97.2	98.4	98.4	96.2	97.7
"	Weibchen 5	97.5	98.4	98.1	97.4	97.2
Sj 5	Männchen 4	98.9	96.7	93.8	95.5	88.4
"	Weibchen 5	98.2	95.7	92.4	92.7	94.0
Sj 6	Männchen 4	98.1	54.5	88.8	86.8	—
"	Weibchen 5	97.5	93.7	90.0	89.4	—
Sj 7	Männchen 4	99.2	90.3	89.6	89.0	89.7
"	Weibchen 5	98.7	87.8	87.2	92.1	92.6
Sj 8	Männchen 4	97.7	89.6	93.4	89.3	90.1
"	Weibchen 5	98.0	89.5	89.9	90.8	87.7

6 Die Ausnutzung wurde jeweils an 5 Tieren bestimmt; wiedergegeben sind die Mittelwerte

1) Group
2) Void value
3) Weeks
4) Males
5) Females
6) Utilization was determined for 5 animals in each case; average values are shown.

Duration of stay in stomach and intestine.

Table 7 shows the oil volumes recovered in the stomach, small intestine, colon and in the stomach + total intestines, 3 and 6 hours after the application of untreated and blown soybean oil (0.2 g/100 cm^2 surface), and the oil resorption resulting from the latter. As can be established, 3 hours after the administration of the soybean oil, 26.6% of the same are reabsorbed, while 37.7% of the untreated oil had already been reabsorbed. This difference (according to the results of the statistical evaluation) should be regarded as highly significant, with $p = 0.001$. When the recovered oil volumes from the stomach and intestine are subsequently compared, it becomes evident that much larger quantities of oil are recovered in the stomach after the administration of blown soybean oil than after untreated soybean oil. On the other hand, no major differences exist in the small intestine and in the colon regarding the recovered oil volumes in both test groups.

Six hours after the oil application the difference between the reabsorption of the two tested oils is even more pronounced. While at that time 93.7% of the untreated soybean oil is reabsorbed, the reabsorption of the blown soybean oil amounts only to 49.5%. During the statistical investigation this difference proved to be highly significant ($p \leq 0.001$). Furthermore, 2 to 3 times more blown soybean oil than untreated soybean oil is found in the stomach, small intestine and colon 6 hours after the oil application.

According to the submitted results, the blown soybean oil under investigation here is reabsorbed at a much slower rate in the rat intestine than the untreated soybean oil. The inhibition of the intestinal motor system, which was detected during the following tests with mice, could possibly be decisively involved here.

TABLE 7

Reabsorption of untreated and blow soybean oil in the rat intestine.

Recovered oil volume in % of the supply.

	1 Magen	2 Dünndarm	3 Dickdarm	4 Magen + Darm	5 resorbierte Ölmenge in % der Aufnahme
6 3 Std. nach Gabe 7 von unbehandeltem Sojaöl	25.5 ± 4.6	32.3 ± 7.4	6.5 ± 1.9	62.3 ± 1.3	37.7 ± 1.5
6 3 Std. nach Gabe 8 von geblasenem Sojaöl	35.7 ± 6.9	35.1 ± 8.1	3.6 ± 1.5	74.4 ± 1.0	25.6 ± 1.5
6 6 Std. nach Gabe 9 von unbehandeltem Sojaöl	7.4 ± 1.9	12.7 ± 3.8	6.3 ± 1.8	26.3 ± 2.3	73.7 ± 2.2
6 6 Std. nach Gabe 10 von geblasenem Sojaöl	19.2 ± 4.9	19.2 ± 2.7	12.0 ± 4.1	50.5 ± 2.9	49.5 ± 2.9

11 Mittelwerte von je 10 Tieren; Ol 010

1) Stomach
2) Small intestine
3) Colon
4) Stomach + intestine
5) Reabsorbed oil volume in % of the supply
6) . . . hours after application
7) of untreated soybean oil
8) . . of blown soybean oil
9) . . of untreated soybean oil
10) .. of blown soybean oil
11) Average values for 10 animals each: oil 010.

Evacuation of fat through the intestinal lymph.

As shown on Illustration 1, the fat content of the intestinal lymph rises rapidly to high values after the application of untreated soybean oil. The maximum is reached after 3 hours. Up to the 6th hour the fat content drop is minimal; the initial value is reached after 24 hours. After the administration of blow soybean oil no lymph fat content increase whatsoever occurs at first. A minor increase is evident only from the 5th hour on. The slow and unsatisfactory reabsorption of blown fats, determinable during the previously mentioned tests concerning the utilization and stay in stomach and intestine of said fats, is confirmed by the available results of tests with lymph-fistula rats. How far modifications related the absorption of auto-oxidized fatty acids into the chylomicrons are involved during the observed phenomenon must be reserved for future investigations.

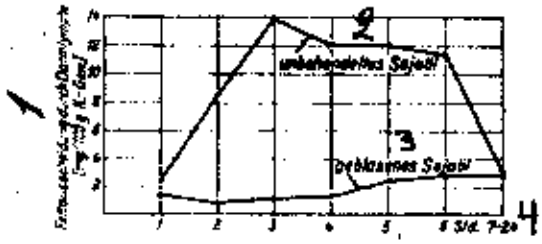

Illustration 1. Fat volume of rats secreted with the lymph after the oral administration of untreated and blown (010) soybean oil (0.25 ml/100 g body weight). (Average values from 6 tests in each case).

1) Fat secretion through the intestinal limph (mg/100 g of body weight)
2) untreated soybean oil. 3) blown soybean oil; 4) 6 hours 7-24

Effect on the motor system of the stomach-intestinal tract.

The following becomes evident from Table 8 which shows the effect of untreated and blown soybean oil on the ink conveyance in the mouse intestine:

TABLE 8

Effect of untreated and blown soybean oil on the ink conveyance through the intestine (oil 010):

1 Behandlung	2 Tierzahl n	3 Tuschetransport in % Darmlänge x̄	±σ
unbehandeltes Sojaöl 4 (0.25 ml/100 g)	10	80.20	± 1.93
unbehandeltes Sojaöl 4 (0.5 ml/100 g)	10	45.61	±10.62
geblasenes Sojaöl 5 (0.25 ml/100 g)	10	28.02	± 8.47
geblasenes Sojaöl 5 (0.5 ml/100 g)	10	7.83	± 8.82

1) Treatment 2) number of animals 3) Ink conveyance in % of intestine length

4) Untreated soybean oil 5) Blown soybean oil.

After blown soybean oil, the ink transportation in the intestine is definitely slower than after untreated soybean oil in corresponding dosages. Accordingly, ink conveyance after the application of 0.25 ml oil/100 g body weight is roundly 80% of the intestine length with untreated soybean oil, and only 28% with blown soybean oil. After the application of 0.5 ml oil/100 g body weight the ink transportation is found to be 46% of the intestine length when using untreated oil, and 8% when using blown soybean oil. It is necessary here, so as to be able to interpret the findings, to be acquanited also with ink conveyance (data) related to animals which did not receive preliminary doses of oil. Such measurements were recently carried out by us; the result was for a group of 10 mice which had not been given any oil, but under otherwise [illegible] test conditions: an ink conveyance of 51.2±2.13% of the total intestine length. When this value is compared to those obtained after oil applications, the following statement can be made concerning the effect on the intestinal activity:

The intestinal activity is definitely stimulated after the application of 0.25 ml untreated soybean oil/100 g, as compared to the controls not loaded with oil; after the application of 0.5 ml untreated soybean oil/100 g no difference is evident, as compared to the control, regarding the intestinal activity; after doses of 0.25 and 0.5 of blown soybean oil/100 g a definite inhibition of the intestinal activity occurs, as compared to the control group which had not received any oil; the inhibition intesnifies with increased oil supply.

The determination of the differences between the corresponding doses of control oil and blown oil is statistically consistent ($p = < 0.001$).

TABLE 9

Cholerese (bile) tests with rats. (Oil 010)

Behandlung	Galle-Ausscheidung in % der Leerperiode ($\bar{x} \pm s_{\bar{x}}$)				
	1.	2.	3.	4.	5. Std. nach Ölgabe
unbehandeltes Sojaöl 0.2 ml/100 g intraduodenal (n = 5)	− 1.42 ± 1.42	+ 2.22 ± 2.22	− 2.88 ± 1.84	− 9.56 ± 0.70	− 15.0 ± 1.40
geblasenes Sojaöl 0.2 ml/100 g intraduodenal (n = 5)	+ 14.26 ± 2.67	+ 15.4 ± 4.34	+ 9.46 ± 4.67	+ 5.24 ± 5.80	− 5.08 ± 2.08

1) Treatment 2) Bile secretion in % of the void period
3) 5 hours after the oil application
4) Untreated soybean oil, 0.2 ml/100 g, intraduodenal (n = 5)
5) Blown soybean oil, 0.2 ml/100 g, intraduodenal (n = 5).

Effect on the bile secretion.

Table 9 shown the effect of untreated and blown soybean oil on the bile secretion of rats. Accordingly, untreated soybean oil (0.2 ml/100 g, intraduodenal) hardly causes any substantial bile secretion changes up to 3 after the oil application. Subsequently, the gall secretion definitely decreases; by the end of the test (5 hrs after the oil application) the secretion is 15% below the value of the preliminary period. The course of the secretion curve is entirely different after the administration of blown soybean oil in corresponding dosages. The bile secretion increases over 15% during the 1st and 2nd hour after the oil was given.

During the following hours the bile secretion drops. During the 3rd and 4th hour after the application of blown oil it is still definitely above the initial value; by the end of the test period the value dropped by -5%, only slightly below the above value. The statistical computation showed that the differences between the two groups are highly significant ($p = \geq 0.001$ for the 1st hour; $p = 0.02$ for the 2nd, 3rd and 4th hours; and $p = \geq 0.001$ for the 5th hour).

These tests did not reveal what causes said stimulation of the bile secretion. The following causes are possible: 1) formation of active substances in this respect during the auto-oxidation, and 2) the choleretic effect could depend on the verified prolonged retention of the blown oil in the stomach and intestine.

Secretion of water, sodium and chloride through the kidneys.

Diuresis-tests with rats show that the i.p. injection of untreated and blown soybean oil to the amount of 0.5 ml/100 g body weight certainly affects the urine excretion. As shown on Illustration 2, urine excretions evidently increase after the oil application during the 1st and 2nd hour of the diuresis-test, as compared to the control group. After doses of untreated oil the excretion increases moderately here, while it increases considerably after doses of blown oil. Since during the following hours of the diuresis test the urine excretions after oil applications approximately match those of the control group, or are slightly higher than the latter, the total excretion after oil applications likewise increases, i.e. it is considerably higher after blown oil than after untreated oil.

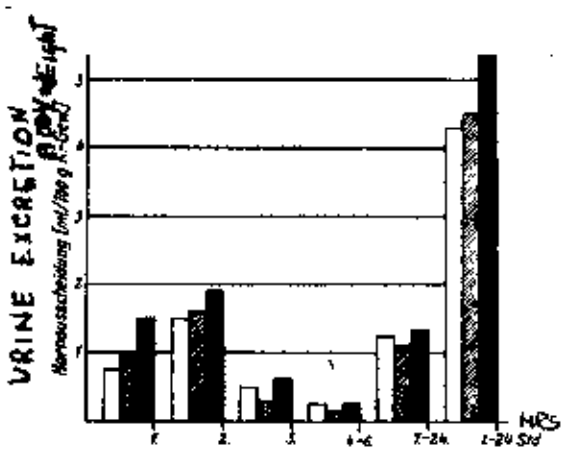

Illustration 2. Urine excretion of rats (average values of 10 animals) after the oral application of 5 ml H_2O/100 g body weight (empty columns) and simultaneous i.p. injection of untreated (shaded columns) and blown (black columns) soybean oil.
Oil dosage: 0.5 ml/100 g body weight/ oil 010.

TABLE 10

Urine, sodium and chloride excretion of rats after the i.p. application of untreated and blown soybean oil.

1 Ausscheidung innerhalb von 24 Std./100 g Körpergewicht

2 Behandlung	3 Harn $\bar{x} \pm s_{\bar{x}}$	4 Na $\bar{x} \pm s_{\bar{x}}$	5 Cl $\bar{x} \pm s_{\bar{x}}$
6 Kontrolle	4.27 ±0.37 ml	0.241 ± 0.031 mÄq	0.193 ± 0.032 mÄq
7 unbehandeltes Sojaöl 0.5 ml/100 g i.p.	4.50 ± 0.14 ml	0.152 ± 0.017 mÄq	0.180 ± 0.015 mÄq
8 geblasenes Sojaöl 0.5 ml/100 g i.p.	5.37 ± 0.29 ml	0.192 + 0.008 mÄq	0.161 + 0.018 mÄq

1) Excretion within 24 hours/100 of of body weight.
2) Treatment 3) Urine 4) Sodium 5) Chloride
6) Control
7) Untreated soybean oil. . . 8) Blown soybean oil . . .

Table 10 shows the average values obtained thereby for the total excretio and their simple and average errors, for each animal group. The statistical evaluation of the results shows that the urine excretions of the control group on the one hand, and those of the animal group treated with blown oil on the other hand differ significantly ($p < 0.05$) from each other. Furthermore, a remarkable difference is likewise evident between the urine excretions of the animal group which received injections of untreated soybean oil on the one hand, and the group which received injections of blown oil on the other hand ($p = < 0.05 > 0.002$). No definite difference exists, however, between the urine excretion volumes of the control group on the one hand, and those of the animal group injected with untreated soybean oil.

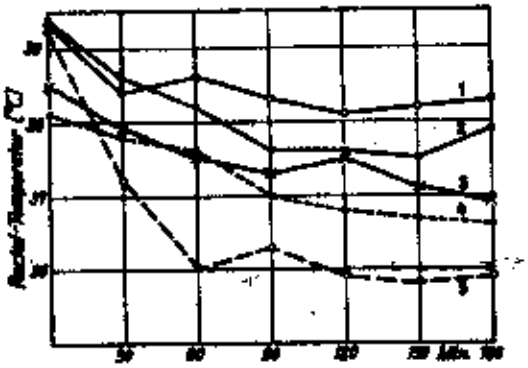

Abb. 5. Rectal-Temperatur von Mäusen nach oraler Gabe von 0.5 ml Sojaöl pro 100 g K.-Gew.

1 Kurve 1: Sojaöl, unbehandelt (Sj KW 1)
2 Kurve 2: Sojaöl, 2 Std. bei 170° C autoxydiert (Sj 1)
3 Kurve 3: Sojaöl, 9 Std. bei 170° C autoxydiert (Sj 5)
4 Kurve 4: Sojaöl, 24 Std. bei 170° C autoxydiert (Sj 7)
5 Kurve 5: Sojaöl, 44 Std. bei 170° C autoxydiert (Sj 8)
6 (Kurven = Mittelwertskurven für n = 10)

Illustration 3. Rectal temperature of mice after the oral application of 0.5 ml soybean oil per 100 g of body weight.

1) Curve 1: soybean oil, untreated (Sj KW 1)
2) Curve 2: soybean oil, auto-oxidized for 2 hours at 170°C (Sj 1)
3) Curve 3: soybean oil, auto-oxidized for 9 hours at 170°C (Sj 5)
4) Curve 4: soybean oil, auto-oxidized for 24 hours at 170°C (Sj 7)
5) Curve 5: soybean oil, auro-oxidized for 44 hours at 170°C (Sj 8)
(Curves: average value curves for n = 10)

The determination of the sodium and chloride-content in the total excreti yielded the highest values for the control group, while the values were considerably lower for the group of animals injected with blown soybean oil. According to the results of the statistical evaluation, however, only the sodium excretion difference between the control group and the animal group injected with untreated oil is to be regarded as significant ($p < 0.05 > 0.02$). On the other hand, no noteworthy differences are determinable when comparing the other animal groups to each other.

Therefore the diuresis-tests with rats indicate that when blown soybean oil is i.p. applied, it affects the diuresis , resulting in a considerable increase of the same. Since secretion of sodium and chloride does not undergo any well-defined changes under these conditions, it can be assumed that the diuresis caused by blown oil is the result of reduced tubular water reabsorption. Further investigations are needed to clarify whether said reduced water re-absorption is of renal origin or whether it is caused by the reduced adiuretine-production of the hypophysis.

Effect on the body temperature.

Mice were used for the tests. The animals received 0.5 ml of the oils at issue per 100 g of body weight, administered with the aid of the esophagus probe. Subsequently, the rectal temperature was taken every 30 minutes for 3 hours with a rectal quick-reading resistance themometer (Manufactured by ATMOS). The temperature was taken in 5 groups including 10 mice each. Group 1, used as control group, received the untreated soybean oil Sj Kw 1; group 2 received the slightly blown soybean oil Sj 1 which proved to be non-toxic during the feeding test; group 3 received the oil Sj 5 which causes considerable growth inhibition but no deaths; groups 4 and 5 received the strongly toxic oils Sj 7 and Sj 8 respectively.

Illustration 3 shows the results. The blown oils caused a drop of the body temperature; it was only slight with the non-toxic oil Sj 1, but the decrease intensified simultaneously with the increased oxidation grade. However, we have tested blown oils as well which failed to affect the body temperature.

Effect on the dextran -edema

Since the suspicion cannot be disregarded that the feeding of auto-oxidized oils can cause symptoms such as a tocopherol-deficiency, we proposed to investigate the question whether malfunctions could be verified, to be interpreted as exsudative diathesis, i.e increased capillary permeability. We therefore investigated whether the dextran paw-edema of the rat is affected. The extent of the dextran-edema (Ill.4) was not affected by the administration of 0.5 ml of an untreated peanut oil per 100 g of body weight. On the other hand, blown peanut oil had a statistically confirmed effect, insofar as it increased the dextran-edema. The effect of a local stimulus is exluded with this testing method. The action mechanism has not yet been clarified. It is subject of debate whether substances form during the auto-oxidations of the oil which have a direct effect on the permeability of the capillarial wall, or whether permeability is indirectly caused by the [illegible] of active substances such as histamine, serotonine or similar [illegible].

It should be mentioned in this context that we found, in the course of other test series with blown soybean oil, that the subcutaneous injection of such oils into the paw of rats causes the formation of an edema. Preliminary treatment of the animals with higher doses of tocopherol inhibits edema formation considerably, but does not prevent

The injection of untreated soybean oil does not cause edema formation. The used blown soybean oil had a peroxide number of 8.4. It is therefore highly unlikely that the locally irritating effect could be caused by the peroxide.

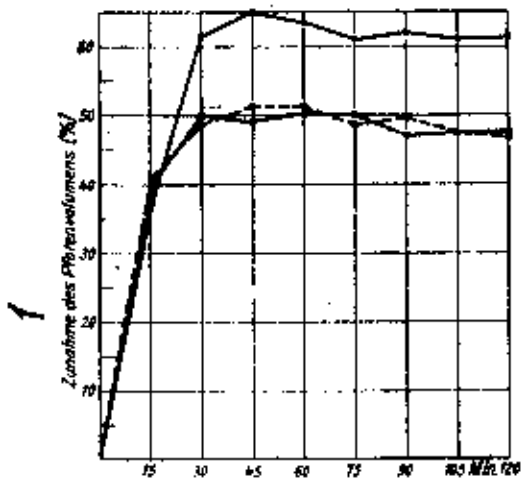

Abb. 4. Intensität und zeitlicher Verlauf des Dextran-Pfotenödems von Ratten

2 O---O ohne Vorbehandlung (Kontrolle)
3 O—O nach oraler Gabe von 0.5 ml unbeh. Erdnußöl/100 K.-Gew.
4 ●---● nach oraler Gabe von 0.5 ml oxyd. Erdnußöl/100 K.-Gew.
5 Ölgabe jeweils 90 Min. vor Dextran-Injektion
6 Mittelwertskurven aus jeweils 8 Einzelversuchen

Illustration 4. Intensity and chronological progress of the dextran-paw edema of rats.

1) Increase of the paw volume (%)
2) . . . without pre-treatment (control)
3) . . . after the oral application of 0.5 ml of untreated peanut oil per 100 kg of body weight.
4) . . . after the oral application of 0.5 ml of oxidized peanut oil per 100 kg of body weight
5) Oil application in each case 90 minutes before the dextran-injection.
6) Average value curves from each of 6 single tests.

Liver function.

Table 11 shows the bromosulfathalein-retention of rats which received fodder with untreated and blown soybeanoil respectively, during 8 weeks.

According to aforementioned findings, the feeding of blown soybean oil causes a well-defined bromosulfathalein-retention in the serum. The effect intensifies simultaneously with intensified treatment of the oil. In all groups which received treated soybean oil, the [illegible] retention is significantly stronger than in the control groups. According to the available findings, the prolonged supply of blown soybean oil is not harmless for the organism, since this evidently inhibits the liver function.

TABLE 11

Bromosulfathalein-test with rats which received blown soybean oil in the fodder.

1 Tiergruppe	2 Behandlung	3 BS-Retention im Serum 20 Min. nach Injektion von 20 mg/kg x̄	±x̄
4 Sj KW₁ n = 9	Futter mit unbeh. Sojaöl 8 Wochen	0.76	± 0.157
5 Sj 5 n = 11	Futter mit gebl. Sojaöl SO 5.2 8 Wochen	2.24	± 0.472
6 Sj 6 n = 11	Futter mit gebl. Sojaöl SO 6.8 8 Wochen	3.48	± 1.120
7 Sj 7 n = 10	Futter mit gebl. Sojaöl SO 10 8 Wochen	4.28	± 1.460
8 Sj 8 n = 9	Futter mit gebl. Sojaöl SO 30 8 Wochen	4.98	± 1.840

1) Animal group 2) Treatment
3) BS-retention in the serum 20 minutes after the injection of 20 mg/kg
4) . . . Fodder with untreated soybean oil - 8 weeks
5) . . . Fodder with blown soybean oil SO 5.2 - 8 weeks
6) . . . Fodder with blown soybean oil SO 6.8 - 8 weeks
7) . . . Fodder with blown soybean oil SO 10 - 8 weeks
8) . . . Fodder with blown soybean oil SO 30 - 8 weeks

Effect of untreated and blown peanut oil on the central excitability of rats.

The tests were performed with rats weighing 100 to 150 g, using the N. GERLICH[10] electroshock-method. Hereby the minimal alternating current voltage was determined first under constant stimulation time- and stimulation frequency conditions (convulsion threshold voltage), which (voltage) can generate a general convulsion during transverse passage through the head. After the administration of the oil the convulsion threshold voltage was determined in intervals of 1 to 4 hours. The convulsion threshold voltages obtained thereby were expressed in percentages of the initial convulsion threshold value and were plotted on the ordinates of a coordinate system proportionate to time as abscissa.

The average convulsion threshold modifications ($\bar{x}$) obtained from 10 single tests in each case and their simple average errors ($s_{\bar{x}}$) were then diagrammatically represented.

An untreated peanut oil as well as peanut oil which had been oxidized for 3 and 5 hours with the method shown below were investigated during these tests.

1 l of peanut oil was in each case transferred in a large round-bottom flask into a thermostatic oil bath, and maintained at a temperature of 150°C. Simultaneously an air jet of 1.5 l per minute was blown through the oil, whereby the stability of the air jet was controlled with a superposed flow meter.

The convulsion threshold curves of Illustration 5 indicate that after the administration of auto-oxidized peanut oil the convulsion threshold curves decline definitely, i.e. the central excitability is intensified under these conditions. The effect becomes obviously stronger with the increasing auto-oxidation rate of the peanut oil.

According to said findings substances are formed during the auto-oxidation of the oil which cause increased central excitability.

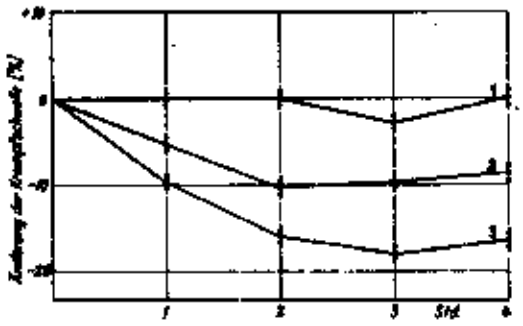

Abb. 5. Änderung der Krampfschwelle bei Ratten nach oraler Gabe von unbehandeltem und geblasenem Erdnußöl (5 ml/kg K.-Gew.)

Kurve 1: Erdnußöl unbehandelt
Kurve 2: Erdnußöl 3 Std. durchblasen
Kurve 3: Erdnußöl 5 Std. durchblasen

Die einzelnen Meßpunkte sind Mittelwerte ($\bar{x}$) aus 10 Einzelversuchen; ihr einfacher mittlerer Fehler ($s\bar{x}$) ist durch die vertikalen Linien dargestellt

Illustration 5. Modification of the convulsion threshold in rats after the oral administration of untreated and blown peanut oil.

1) Modification of the convulsion threshold (5)
2) Curve 1: peanut oil, untreated
3) Curve 2: peanut oil, blown through for 3 hours
4) Curve 3: peanut oil, blown through for 5 hours.
5) The single measuring points are average values ($\bar{x}$) from 10 individual tests; their simple average error ($s\bar{x}$) is represented by the vertical lines.

Effect of untreated and blown peanut oil on the mobility of rats.

Rats weighing 100 to 150 g were used for the tests. The mobility of the animals was measured in vibrating cages which were built as follows: the cages were suspended on spiral springs; the slightest movement triggered contact shots. The contact shots were registered with the aid of telephone computers.

The tests were carried out with 6 groups including 8 rats each.
Group 1 received untreated peanut oil (0.5 ml/100 g)
" 2 peanut oil, which had been auto-oxidized for 1 hour
" 3 " " " 2 hours
" 4 " " " 3 "
" 5 " " " 4 "
" 6 " " " 5 "

The oil was treated as specified earlier (central excitability). After the application of the oil a waiting period of 30 minutes followed as a rule before starting the activity registration, so as to let the animals get accustomed to their new environment and to the cages. The activity was subsequently registered during 5 hours. While the measurements took place the animals were kept in a small, quiet room; its temperature amounted to 26°C.

Table 12 shows a compilation of the test results; the latter were evaluate according to the t-test of STUDENT.

TABLE 12

Rat activity after the oral administration of (0.5 ml/100 g) untreated and blown peanut oil.

Rattenaktivität nach oraler Gabe (0.5 ml/100 g) von unbehandeltem und geblasenem Erdnußöl

1 Erdnußöl	2 Kontakte/5 Std. $\bar{x}$	$s_{\bar{x}}$	p	
3 unbehandelt	2131	± 269	—	—
4 1 Std. geblasen	2455	± 263	< 0.50 >	0.40
2 " "	2689	± 349	< 0.30 >	0.20
3 " "	3104	± 340	< 0.05 >	0.02
4 " "	3197	± 241	< 0.02 >	0.01
5 "	3548	± 344	< 0.02 >	0.01

5 Mittelwerte von jeweils 8 Tieren

1) Peanut oil 2) Contacts/5 hours
3) untreated
4) blown for 1 hour.
5) Average values for 8 animals in each case.

According to the submitted findings mobility is obviously stimulated following the application of blown peanut oil. Said effect intensifies simultaneoulsy with the increased auto-oxidation of the oils, i.e. the prolonged blowing of the same. Table 12 furthermore indicates that the activity of the animals shows significant differences--as compared to the control group--after having received oils which had been blown for 3 hours or longer.

The test results obtained here coincide well with the findings of the electroshock-tests, and therefore likewise prove that substances with centrally stimulating effects occur in the blown oils.

Effect on the cell metabolism.

For these tests, homogenates, isolated mitochondria and the cyclophorase system of the rat liver served as enzyme-preparations. Specific data on the preparation of the above are found under Methodology. 1 to 2% emulsions, prepared in water or tris-buffers with or without addition of tween 20 had no effect with any of the tested blown soybean oils on the respiratory utilization (Veratmung) of the following substrata: citrate, -ketoglutarate, succinate, malate and L-glutamate. The no-load respiration of the ingredients without added substratum likewise remained unaffected. Accordingly, oils blown at higher temperature do not affect cell respiration in vitro.

It became evident, on the other hand, that the volatile fraction distilled during the blowing of oils contains substances which inhibit cell metabolism. Evidence was submitted from various sides, proving that a large number of homologous alkans, monoenals and dienals are contained in said fraction, i.e. substances with very strong reactivity[20-24]. This volatile fraction showed a strong inhibiting effect on the respiratory utilization (Veratmung) of the citric acid cycle links of fatty acids (capronate) and of L-glutamate. For specific data see Table 13.

The saturated aldehydes can probably not be held responsible for said effect. Hexanal, large volumes of which form during the auto-oxidation of fats, was investigated by us as a pure substance and failed to show any such effect. During the test it was partially oxidized by the enzime-preparations.

TABLE 13

Inhibition of cell respiration by the fraction of volatile substances which form during the blowing of soybean oil.

1 Substrat	2 Enzympräparat (Rattenleber)	3 Zahl der Versuche	4 Beobachteter Effekt % Hemmung	% Aktivierung
Citrat	Cyclophorase	1	28	—
α-Ketoglutarat	Cyclophorase	4	50, 86, 100, 100	—
Succinat	Cyclophorase	1	46	—
	Mitochondrien	1	85	—
Fumarat	Cyclophorase	1	95	—
Malat	Cyclophorase	4	14, 37, 64, 97	—
L-Glutamat	Cyclophorase	2	21, 35	—
	Mitochondrien	2	100, 100	—
Capronat	Cyclophorase	1	100	—
5 endogene Substrate (Leeratmung)				
	Cyclophorase	7	43, 44, 45, 46, 51, 63, 100	—

6 Je Ansatz wurden 0.05 ml des Destillats zugesetzt

1) Substratum
2) Enzyme preparation (rat liver)
3) Number of tests
4) Observed effect
% inhibition - % activation
5) endogenous substratum (no-load respiration)
6) 0.05 ml of the distillate was added per test solution.

E. SCHRAUBENSTEIN and collaborators demonstrated that water-soluble peroxides form during the digestion of polyene acids with water at low temperatures and with air access; said water-soluble peroxides inhibit the respiration and glycolysis of tumor cells as well as the fermentation of yeast. The authors were able to show that the metabolism inhibitions are presumably caused by the deactivation of SH-enzymes. Various reports have been submitted early on the inhibition of SH-enzymes or destruction respectively, of SH-groups in proteins by peroxides.

The absence of cell metabolism inhibitions during our tests, as compared to the findings of E.SCHAUENSTEIN and collaborators, is explainable as follows: the oils blown by us at high temperatures practically do not contain any peroxides.

Organ weights.

Table 14 shows the organ weights of animals fed with the toxic blown soybean oils. The organ weights, expressed in percentages of the body weight, are higher for the animals fed with blown soybean oil than (the organ weights) of the controls. We suspect that this merely indicates reduced organ weights as a consequence of growth inhibition; therefore no direct effect of the blown oils on the organ weights can be presumed. It seems necessary to us, however, that supplementary investigations be undertaken to clarify the question at issue here.

Histological examination.

Animals were principally used for histological examinations which had received the toxic blown oils Sj 5 to Sj 8 in the course of the feeding tests. The groups Sj kW served for control purposes. The following organs were examined: heart, liver, kidney, spleen, small intestine, colon and testicles, furthermore also the suprarenal glands and the thyroid glands. The organs were fixed in 5% formaline. The prepared paraffin sections were dyed with hematoxyline-eosine and with scarlet. The heart muscle system of all animals displays a clearly determinable transverse stripe pattern. The (cell) nuclei are oval-shaped and the nucleus chromatin has a fine structure. The anatomical structure of the liver parenchyma is normal. The connective tissue around the larger vessels and around the GLISSON triangles is not enlarged. The protoplasm of the liver cells is finely granulated and the nuclei have a fine chromatin structure. With the scarlet dye the content of the liver capillaries of some animals becomes reddish, but no difference exists in most cases between the control- and the test animals.

In the kidney tissue numerous incorporations are visible which become dark violet with the H.E. coloring. Said incorporations are more frequently evident in those animals which received strongly oxidized fat as basic feed than in those which were fed slightly oxidized fats. In most animals the incorporations are located within the intermediate zone of the marrow substance, and the first incorporations were detected in the collecting tube epitheliums.

TABLE 14

Organ weights of rats fed with blown soybean oil.

1 Gruppe	2 Körpergewicht in g	3 Darmtrakt	4 % des Körpergewichtes Herz 5	Leber 6	Milz 7	Niere 8	Testes 9
Männchen 10							
Kontrolle 11							
Sj KW 1	248	7.33	0.41	3.16	0.25	0.67	1.38
Sj 5	129	15.17	0.63	6.16	0.40	1.08	2.11
Sj 6	109	15.60	0.62	7.23	0.43	1.16	1.87
Sj 7	112	18.45	0.65	7.51	0.42	1.26	2.04
Sj 8	103	19.83	0.61	7.33	0.43	1.41	2.50
Weibchen 12							
Kontrolle 13							
Sj KW 1	206	11.87	0.41	3.27	0.28	0.67	—
Sj 5	112	14.60	0.61	6.32	0.42	1.05	—
Sj 6	87	18.29	0.73	7.65	0.47	1.44	—
Sj 7	112	18.44	0.59	7.47	0.46	1.30	—
Sj 8	94	16.93	0.70	7.41	0.42	1.15	—

14 Angaben in % des Körpergewichtes; Mittelwerte

1) Group
2) Body weight in g
3) Intestinal tract
4) % of body weight
5) Heart
6) Liver
7) Spleen
8) Kidney
9) Testicles
10) Males
11) Controls
12) Females
13) Controls
14) Data in % of body weight; average values.

In cases with larger incorporations these begin to form rank growths in the tubulus lumen, and numerous mitoses indicate an intensive cell regeneration. The basally positioned kidney epitheliums of the incorporations later die. The incorporations are therefore located between the still preserved basal membrane or the interstitial connective tissue respectively and (between) the regenerated kidney epitheliums. As a consequence of large-scale incorporations, the epitheliums in the surrounding area are destroyed by the pressure to which they are subjected. Part of the incorporation can then be transferred into the lumen of the small urethra. Most of it, however, remains at the original site. These masses can reach a size of 200 μ and their size frequently causes an occlusion of the urethra.

The occlusion results in the damming up of the primary urine and in some preparations also causes a widening of the free space of the kidney corpuscles. As a rule, the tissue incorporations are nonreactive; only occasionally are marginal accumulations of neutrophile granulocytes found. An accurate histological examination remains subject to further study.

The microscopic image of the spleen parenchyma likewise shows a normal histological structure. The follicles are clearly evident in the preparations, and the pigment content and phagocytes within the red pulpa are not increased. No inflammation-caused changes are detectable in any of the animals during the histological examination of the stomach intestinal tract.

The testicle parenchyma of the control animals as well as of the test animals is free of pathological-histological changes. In the small collecting tubuli the basal cells with their large chromatin-poor nuclei shaped like small bubbles can be clearly distinguished from the sperm-cells proper. The chromatin-rich, spherical nuclei of the spermatogoniums and spermatozids are frequently in a mitotic condition-- in the control animals as well as in the test animals. Numerous spermines (Spermien) are located in the collecting tubuli; their microscopic picture shows no pathological changes.

The thyroid glands of control animals (Sj Kw) and of 10 animals from Group Sj 8 were examined. The thyroid glands of the last-mentioned animals showed activation. For the purpose of determining the differences, the thyroid glands were measured according to the method of U. UOTILA and O. KANNAS[30]. The average epithelium component % of the control animals was 35.4%; but it was 51.3% for the animals of the group Sj 8.

The examination of the surparenal glands likewise involved the glands of 10 animals each from the control group Sj Kw and from the group Sj 8. The histological aspect of the suprarenal glands of the animals from the control group was normal. The zona glomerulosa was well-defined, and the zona fasciculata contained numerous so-called stationary cells the protoplasm of which became reddish when dyed with H.E.; their nuclei were smaller than those of the adjacent active cells.

A minor progressive transformation became evident in the animals from Group Sj 8. No stationary cells could be found in the zona fasciculata. For the purpose of verifying said progressive transformation a number of nuclei in the external zona fasciculata was counted and placed on an area of 0.225 mm^2 of the histological sections. Furthermore, the nuclear volume of 100 nuclei each from the zona fasciculata was measured and the average nuclear volume was computed. In addition to the above, the cumulative curve of the counted nuclei was plotted according to E. HINTZSCHE[31]. The values of Group Sj thereby coincided fully with those of the control group.

TABLE 15

Findings in cell nuclei from the zona fasciculata of the suprarenal gland cortex.

	1 Kontrolle Sj KW	2 Gruppe Sj 8
3 Zahl der Zellkerne in 0.225 mm^2	91.5	80.5
4 Durchschnittliches Kernvolumen in μ^3	151	132

[30] Acta endocrinol. 11, 49 [1952].
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1) Control Sj KW
2) Group Sj 8
3) Number of cell nuclei volume in μ^3
4) Average nucleus volume in μ^3.

Discussion

The findings reported earlier show that preparations are obtained by blowing oils under adequate conditions; these preparations have a certain toxicity which cannot be traced back to the presence of peroxides. We made every effort to clarify the mechanism by means of which the blown oils activate their toxic effect. In the course of said investigations we secured numerous findings requiring pathological evaluation, but neither the testing of the cell metabolism, nor the histological examination of the organs, nor the investigation of the organ functions yielded any indication concerning the immediate cause of death when animals succumb to the toxic effect of blown oils after a brief feeding period. Nevertheless, certain pathological diagnoses could be made, such as disturbances of the water- and mineral metabolism of the liver function, the functioning of the central nervous system, modifications of the secretion output, of the muscular (motor) performance the performance of the digestive tract as well as the effect on the functioning of the thyroid glands, with the resulting consequences. In a previous study from our Institute, [illegible] and K. [illegible][32] showed that modifications of the lipid metabolism likewise occur, i.e. all lipid fractions, including cholesterin, are reduced in the plasma; they furthermore showed that the polyenic acid content of the stored fat, as well as of the organic fat decreases considerably. But all these findings do not suffice as an explanation of the high mortality rate or of the substantial growth inhibition after the feeding of the strongly toxic preparations.

Our tests demonstrated that while high doses of tocopherol do not prevent the toxic effect of the blown oils, they [illegible], weaken said effect in some cases. Furthermore, it was [illegible] that the feeding of blown oils causes a certain deficiency of essential fatty acids in the organism. It should therefore be [illegible] part of the symptoms could be attributed to [illegible] deficiency of the organism and also to an absolute [illegible] tocopherol deficiency.

Various laboratories, including our own, among others[32], recently demonstrated that the body fat of humans and animals contains substances which are colored again by decolorized dichlorphenol-indophenol, react positively and show other similar reactions to the thiobarbiturate-test; these substances are customarily defined as "fatty (aliphatic) peroxides". We proved that such substances are stored in the organism after the feeding of blown oils[32]. Organ-homogenates or isolated mitochondria can oxidize fat in vitro into such "peroxides", i.e. into substances which react as indicated above. This presumably occurs in the form of a non-enzymatic reaction.[32-34]

The extent of said reaction unequivocally depends on the extent of the preliminary tocopherol-supply.[35] After a plentiful tocopherol application the "peroxide-formation" by means of homogenates or mitochondria is minor. This also coincides with our in vitro findings. It is therefore likewise possible to trigger serious tocopherol deficiency conditions with high doses of polyenic acids (for example: by feeding fish oils) when the tocopherol reserves of the organism are low. This can trigger, for example, chicken encephalomalacie[27-30].

The feeding of blown oils undoubtedly increases the tocopherol-need of the organism and can cause tocopherol deficiency. This conclusion can well be reached from feeding test 3 described in present study. The reduction of the polyenic acid reserve of the organism (also verified by us) which (reduction) is more pronounced in the organic fat than in the stored fat, is comprehensible as a consequence. The inadequate linolenic acid supply of the organism is excluded as possible cause according to our test system. Showing nevertheless reduced the dien-acid content of the soybean oil used as feed to [illegible] when the supplied feed volume is taken into consideration, a daily dose of 300 mg linolenic acid still results per rat, i.e. [illegible] of the amount needed.

Despite the high supply volume the animals were unable to maintain the lynolenic acid reserve of their organism. During this test the initial trien-acid increase in the organic fat did even the [illegible] and incipient deficiency condition concerning the essential fatty [illegible] however, during the intake of a 15-fold volume of the normal d[illegible] need said condition could not have been due to external [illegible] instead, its origin must have been endogenous.

Some of the findings mentioned in present work also refer to an essential fatty acid deficiency as a possible cause. We believe that the increased water intake of the animals, the increased capillary permeability (dextran-edema) and the diuresis intensification should be evaluated in this context. Admittedly, other findings seem to contradict the above supposition, specifically in view of the missing testicle atrophy. It must be considered here, however, that testicle atrophy as a consequence of essential fatty acid deficiency is not an early symptom; it occurs only when the deficiency reaches a high degree; therefore the feeding period during our tests might not have been long enough to trigger a testicle atrophy. Our polyenic acid analyses of the body fat[32] likewise show that no serious deficiency condition could be present.

We therefore believe that a relative polyenic acid deficiency should be taken into consideration ethologically as well, together with the consequences resulting from the feeding of large quantities of blown oils; but it should be regarded as a secondary factor only. As stated above, the principal mechanism of the toxic effect has not yet been clarified. Our efforts aimed at its clarification continue.

The concept "toxicity" requires an explanation in this context. During Feeding Test 3 we attempted to determine the minimal dose under which harmful effects (mortality and growth inhibition) can no longer be expected. We reached the conclusion on the basis of our results that said minimal dose is slightly below 2.1 of the feed weight, i.e. it amounts approximately to 1 to 2 . This, however, means a daily dose of 150 to 300 mg for an adult rat, equal to 0.15 to [illegible] weight. Referred to a human weighing [illegible] kg, the daily blown fat [illegible] would amount to 31.5 to 63 g. the toxicity of issue is accordingly not especially high. This indicates that damage resulting in humans from blown oils is not excessively important in practice. The fact should not be overlooked that our tests as well as the tests reported in literature are performed under conditions which are considerably exaggerated as compared to daily nutritional practice: this applies to the treatment of the fats as well as to the administered dose. However, it is occasionally necessary, for the clarification of the mechanism of biological effects, to work under [illegible] conditions which do not exist in everyday life.

Summarizing, the following statement can be made:
By blowing oils rich in polyenic acid at higher temperatures, preparations are obtainable the toxicity of which first increases simultaneously with intensified blowing; it reaches a maximum and subsequently drops again. Said drop is presumably due to the fact that the reabsorption capacity of the substances which are responsible for the toxicity gradually deteriorates. The toxicty of said blown oils is not conditional to the peroxide content but can probably be traced back to the oxipolymers. The limiting dose from which on the toxic effects of such blown oils must be reckoned with was determined in the rat as approximately 0.45 to 0.90 g per kg of body weight.

The following effects of blown oils were established: growth inhibition connected with a considerable deterioration of the feed- and albumen efficiency, occasionally high mortality rate, disturbances of the liver function, increased central excitability, intensified spontaneous mobility of the animals, inhibition of the motor system in the stomach-intestinal tract, modifications of the kidney function, increased capillary permeability, modifications of the motor performance and secretion output in the stomach-intestinal tract, histologically determinable changes of the thyroid gland as well as incorporations (deposits) in the kidney. But neither the histological examination nor the investigation of organ functions, nor the testing of the [illegible] metabolism clarified the mechanism through which the blown oils can have a lethal effect. The immediate cause of death of the animals as a consequence of blown oil feeding could not be determined by us to date.

Our tests indicate that the feeding of blown oils increases the essential fatty acid need of the organism, and that the effects of the blown oils could presumably be interpreted as the consequence of a relative deficiency in the above-mentioned nutrient factors; but these are merely secondary factors within the overall problem concerning the physiological effects of blown oils.

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Observation on Lipid Utilization in Hens Fed Vegetable and Animal Fat Supplemented Diets[1]

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THE recent advent of the synthetic detergent industry has created a surplus of inedible animal fats making them, thereby, available to the feed industry at a cost permitting their competition with carbohydrate energy sources (Ewell, 1953; and Krieg, 1954). The addition of these fats to feeds, however, has been made without serious consideration of the biochemical and physiological changes they might induce. Human studies indicate that an abnormal lipid metabolism may be induced by a large intake of saturated fat, (Ahrens *et al.*, 1954, 1957; and Beveridge, 1956) whereas no such effects occurred when unsaturated fat was consumed. Similar observations in the mature chicken upon feeding saturated animal fat have been reported by Weiss and Fisher (1957) and Price *et al.* (1957).

Although the effect of feeding an unsaturated fat to hens has been studied by

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TABLE 1.—*Experimental design*

Fat supplementation	Protein level	
	15%	25%
None	lot 1	lot 2
Animal fat, 10%	lot 3	lot 5
Corn oil, 10%	lot 4	lot 6

several workers in connection with studies not related to the present problem (Heywang, 1943; Walker, 1951; Reiser, 1950) no one has compared saturated vs. unsaturated fat in a long term study of their utilization by chickens. The object of the present report is to present data collected during the first year of such a long term study comparing an unsaturated fat (corn oil) with a saturated one (animal fat).

EXPERIMENTAL PROCEDURE

Two fats were studied, animal fat (yellow grease) and corn oil, together with a control group receiving no added fat. Each treatment was further subdivided as shown in Table 1. The composition of the experimental diets is presented in Table 2. The elevated levels of vitamins A and D and of calcium were supplied in an attempt to overcome the problem of "cage fatigue", a decalcified condition often observed in cage layers.

The birds used in the experiment were Single Comb White Leghorn pullets from college farm stock. Chicks were reared to 12 weeks of age on a non-fat supplemented starting ration and then received the experimental rations. Twelve birds were allotted to each treatment and maintained in growing cages from 12–17 weeks and were then moved to individual laying cages. The cages were in a temperature regulated room and artificial lights were maintained from 4 a. m. to 8 p. m. providing a 16-hour light day from 12 weeks of age. Feed was supplied *ad libitum* and a continuous supply of water was available to the birds. Although the experiment is still being continued this report summarizes the data collected during the first year on the six dietary regimes.

Individual daily egg records were maintained for all birds. All eggs were weighed

TABLE 2.—*Experimental diets*

Ingredients	Diet composition %					
	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
Yellow corn meal	62	42	52	52	32	32
Soybean oil meal (50% protein)	20	40	20	20	40	40
Corn oil[2]	—	—	—	10	—	10
Animal fat[3]	—	—	10	—	10	—
Basal diet[1]	18	18	18	18	18	18
Calculated Composition						
Protein (N×6.25)	17	26	16	16	25	25
Calcium	2.68	2.75	2.68	2.68	2.75	2.75
Phosphorus	1.16	1.25	1.13	1.13	1.23	1.23
Metabolizable energy Cal./lb.	1,242	1,163	1,420	1,480	1,342	1,410

[1] The basal diet was composed of the following (values are expressed as percentages of the complete diet): dehydrated alfalfa 3%, dried whey 2%, corn distiller's solubles 2%, butyl fermentation solubles 2%, dicalcium phosphate 4%, limestone 1%, trace mineral mix 3% (Mico concentrate, Limestone Corporation of America, Newton, New Jersey), salt 0.5%, choline chloride (70%) 25 gm./100 lbs. of diet, vitamin A & D premix 100 gm./100 lbs. of diet (furnished 6,600 I.U. vitamin A and 1,100 I.C.U. vitamin D_3/lb. of diet), vitamin B_{12} antibiotic supplement 0.5%, DL-methionine 0.1%.

[2] Edible corn oil, Staley Manufacturing Company, Decatur, Illinois.

[3] Yellow grease, Standard colors of the Fat Analysis Committee of the American Oil Chemist's Society (FAC) color, 37–39; free fatty acids (FFA), 15–20%; moisture and unsaponifiable material (MIU), maximum 2.0; activated oxygen method (AOM), value not less than 20 hours.

daily for the first 2 months of production and for a 5-day period at the end of each month thereafter. Birds were weighed at approximately 6-week intervals. Monthly feed records were kept for each lot. Mortality was recorded and each bird that died was examined by the Experiment Station pathologist.

Iodine numbers and the sterol content of the egg fat were determined at approximately 6-week intervals. Four eggs were selected randomly from each lot, the yolks were separated from the whites, and the four yolks from each lot pooled. An aliquot of the pooled yolks was extracted with a 2:1 chloroform methanol mixture. An aliquot of the extract was taken for the iodine number determination as described in the A.O.A.C. (1955) method. Another aliquot was taken to determine the sterol content by a modification of the method of Zlatkis *et al.* (1953).

Individual blood samples from the wing vein were taken at intervals of 4–6 weeks. A heparinized syringe was used to prevent coagulation and the samples were centrifuged for 20 minutes at 2–3,000 R.P.M. to separate the plasma and cells. Aliquots (0.1 ml.) of plasma were taken for cholesterol and total lipid determinations. After the first four determinations a highly significant ($P<0.01$) positive correlation was found between plasma cholesterol and total plasma lipids ($r=0.618$). In view of this observation the total lipid determination was discontinued. The first four determinations were made on 20 birds only, four birds per lot with the exception of lot 2. Thereafter all birds were sampled. The cholesterol in plasma was determined by the method of Zlatkis *et al.* (1953) and the total lipid by the method of Huerga *et al.* (1953).

Analysis of data was accomplished by the analysis of variance as described by Paterson (1939) for factorial designs; where a significant F value was obtained the significant differences between treatments were determined by the multiple range test of Duncan (1955).

RESULTS

All data are summarized in Table 3. No statistically significant differences were found in days to the deposition of first egg, egg production, egg size or feed efficiency.

The iodine number of the egg fat from birds receiving corn oil was significantly ($P<0.01$) higher than that of eggs from birds receiving either the control or animal fat diets. This was anticipated from the findings of Cruickshank (1934). The sterol content of the egg fat was higher in the groups receiving corn oil though not significantly so.

TABLE 3.—*Effect of dietary fat and protein on egg production, egg fat composition, and plasma cholesterol in the hen**

Measurements taken	Lot 1 15% protein No fat	Lot 2 25% protein No fat	Lot 3 15% protein 10% an. fat	Lot 4 15% protein 10% corn oil	Lot 5 25% protein 10% an. fat	Lot 6 25% protein 10% corn oil
Days to 1st egg	155 ± 2.9	158 ± 2.8	157 ± 2.8	159 ± 3.1	149 ± 3.1	152 ± 4.1
Egg/bird/month	20.4 ± 1.5	20.2 ± 1.5	19.3 ± 1.3	17.9 ± 1.5	20.1 ± 1.3	20.3 ± 1.2
Egg weights (gms.)	55 ± 1.5	54 ± 1.7	58 ± 1.7	58 ± 1.8	56 ± 1.6	56 ± 1.6
Kg. feed/doz. eggs†	2.2 ± 0.14	2.1 ± 0.11	2.1 ± 0.15	2.0 ± 0.17	1.8 ± 0.10	1.8 ± 0.10
Kg. feed/bird/mo.†	3.73 ± 0.04	3.80 ± 0.06	3.42 ± 0.08	2.94 ± 0.10	3.18 ± 0.02	3.14 ± 0.10
Body wt. (lbs.)	4.4 ± 0.07	4.1 ± 0.03	4.6 ± 0.08	4.4 ± 0.08	4.4 ± 0.05	4.2 ± 0.05
Iodine No. of egg fat	69 ± 1.0	71 ± 1.0	68 ± 1.5	80 ± 2.7	69 ± 0.7	82 ± 2.5
Sterol content of fat (mg./gm.)	56 ± 2.9	61 ± 2.4	59 ± 2.3	63 ± 2.1	58 ± 2.5	65 ± 3.0
Plasma cholesterol (mg./100 cc.)	195 ± 13.0	204 ± 15.4	212 ± 14.1	205 ± 15.2	230 ± 17.9	203 ± 13.8

* All values represent mean ± their standard errors.
† Feed values calculated from monthly group data.

The birds receiving corn oil consumed less than the groups fed animal fat which in turn consumed less than the control groups. Assuming that feed consumption is regulated by the caloric density of the diet, these observations would indicate a higher caloric value for corn oil as compared to animal fat. This is supported by the recent report of Renner and Hill (1957) which showed corn oil to have a higher caloric value than animal fat, 8.62 and 7.32 Calories of metabolizable energy per gram respectively. This report also indicated a better utilization for corn oil (91.2%) as compared to animal fat (78.4%). The protein levels used in this study exerted no apparent effect on feed consumption attributable to protein per se; the low protein groups did consume less feed in all but one instance. This was obviously a reflection of the higher energy content of corn as compared to soybean oil meal, since soybean oil meal was added at the expense of corn to obtain the higher protein level.

The groups receiving animal fat were significantly ($P<0.01$) heavier than either the corn oil or control groups. The birds on the lower protein level were also significantly ($P<0.01$) heavier than the high protein groups.

The mortality in the animal fat groups was higher but since the mortality of all groups combined was only 10%, it would seem unwise to attribute any significance to this observation. The number of birds which died in each lot was as follows: lot 1 (1), lot 2 (0), lot 3 (3), lot 4 (1), lot 5 (1), lot 6 (0). In addition one bird from lot five was sacrificed shortly after the initiation of the study. Of the birds which died one was found to be an internal layer and the cause of death was presumably attributable to the toxic products of the deterioration of the eggs in the body cavity; two birds showed no evident cause of death, and the remaining three birds exhibited symptoms of the cage fatigue syndrome described by Couch (1955).

The one bird in the high protein-animal fat group (lot 5) which was sacrificed was found to have a normal plasma cholesterol level (132 mg.%) at the start of the experiment (12 weeks of age). After less than 2 weeks on the animal fat diet the plasma cholesterol level had increased to over 10 times the initial value (1332 mg.%). Upon post mortem examination this bird showed an enlarged and fatty liver. These observations denote a deranged lipid metabolism.

The plasma cholesterol levels of the animal fat groups were higher than those of any other group; under the conditions of this experiment this difference did not reach statistical significance.

A significant ($P<0.05$) negative correlation ($r=-0.314$) was found between egg production and the plasma cholesterol level. However, when the birds were divided into high and low groups on the basis of egg production, a "t" test failed to show significance ($P<0.20$) in the plasma cholesterol values of the two groups.

No differences, other than those previously mentioned for body weights, were attributable to the varying protein levels, indicating that both levels were adequate.

DISCUSSION

The cumulative evidence concerning the metabolism of animal vs. vegetable fat by the laying hen indicates a difference in utilization between these fats.

Abnormal fat accumulation, particularly with regard to liver fat, is a well recognized symptom of various physiological and pathological disturbances (Benton *et al.*, 1956; Wohl and Goodhart, 1955). The greater body weight of the animal fat groups as compared to the corn-

trol lots but particularly when compared to the corn oil groups appears to indicate an abnormal fat deposition induced by dietary animal fat. This interpretation is further strengthened by the large acumulation of liver fat in the sacrificed bird. The condition observed in this bird is similar to the fatty liver condition reported by Weiss and Fisher (1957) and Couch (1956).

An elevation of the plasma cholesterol levels of the animal fat groups and a possible reduction in the corn oil lots might have been anticipated from published reports. Weiss and Fisher (1957) in their recent study observed significant increases in the plasma cholesterol levels of birds receiving animal fat. Studies in the human have shown increases in plasma cholesterol from the addition to the diet of animal fat (Beveridge *et al.*, 1956; and Ahrens *et al.*, 1957). These same workers also demonstrated a plasma cholesterol lowering effect for corn oil. In a recent report Jones *et al.* (1957) presented evidence for the effectiveness of corn oil in preventing hypercholesterolemia in the cholesterol-fed chick.

That the higher plasma cholesterol levels of birds on the animal fat diets did not attain significance may be associated with the large individual fluctuations encountered in the hen in contrast to that observed in the rooster. This may be attributed to the hormonal differences between the hen and rooster, or mature individuals of other species. Since the hen is in a continuous state of reproduction, the plasma cholesterol level is considerably influenced by the endogenous estrogen secretion (Stamler *et al.*, 1954). Confirmation of the importance of hormonal influence is demonstrated in Table 4 which shows the relationship between plasma cholestreol level with age. Table 4 presents data from 20 birds for which plasma cholesterol data were available from the start of the experiment. It can be seen that the plasma cholesterol level increases significantly ($P < 0.01$) with the onset of egg production and continues to fluctuate significantly between sampling periods until 300 days of age. For a more complete discussion of these observations see Leveille *et al.* (1957).

TABLE 4.—*Relationship of plasma cholesterol to onset of egg production*

Age of birds (days)	Plasma cholesterol* (mg.%)
84	116 ± 2†
105	152 ± 2
133	223 ± 31
168	285 ± 24
198	208 ± 15
232	238 ± 16
273	239 ± 21
317	192 ± 12
365	182 ± 13
414	202 ± 10
448	212 ± 23

Average age to deposition of initial egg—155 days.
Average age to 100% production—172 days.
* Mean values for 20 birds.
† Standard error of the mean.

Several factors in addition to hormonal influence may exert, singly or in combination, an effect on the plasma cholesterol level; particularly to offset the effect of animal fat. Ahrens *et al.* (1957) reported that, in the human, dietary fat had little effect until it constituted 40% of the caloric intake. Since the fat in the diets used in the present study constituted only 20–25% of the caloric intake, the level of fat may not have been high enough to exert its full effectiveness.

A masking effect by protein may also be partially involved in the smaller plasma cholesterol response. In recent studies both Kokatnur *et al.* (1957) and Nikkila and Ollila (1957) report that the plasma cholesterol level is affected by dietary protein in the region below the minimum protein requirement. Kokatnur *et al.* (1957

also found dietary fat to be effective only when the protein level was inadequate.

The significant negative correlation noted between egg production and the plasma cholesterol level, together with a non-significant difference in the plasma cholesterol levels of the high and low egg production groups, can be considered as further evidence of a derangement in fat metabolism due to dietary animal fat. Since no relationship between egg production and the plasma cholesterol level of birds on non-fat supplemented diets has ever been shown, but a significant negative correlation was observed by Weiss and Fisher (1957) for hens fed animal fat, it appears that lowered egg production accompanies the higher cholesterol levels attributable to dietary animal fat but not the egg production of birds with a normally high cholesterol level.

Considerable emphasis has been placed in this discussion on interpreting the differences observed between the fats studied. It would not be complete, however, without pointing to the absence of improvements in egg production and feed utilization due to fat-supplementation regardless of source. In view of this important finding and the strong possibility that animal fat is not properly utilized, its addition to practical laying rations would not appear warranted.

SUMMARY

Single Comb White Leghorn hens were fed rations containing no fat or 10% levels of corn oil and tallow, each at levels of 15 and 25 percent protein from twelve weeks of age. No significant differences were observed in days to initial egg, egg size, egg production, or feed efficiency.

The birds receiving animal fat were heavier than either the controls or corn oil-fed groups. The birds on the low protein diets also exhibited higher body weights than the birds on the higher protein level. The birds fed animal fat had a higher plasma cholesterol level which contributed to a significant negative correlation between egg production and plasma cholesterol level. Mortality was low in all treatment groups and there were no significant differences.

The iodine number of the egg fat from birds receiving corn oil was significantly higher than the animal fat or control groups. The corn oil-fed birds also had a higher but non-significant amount of sterol in the egg fat.

The cumulative evidence obtained in the present study points toward major differences between the utilization of corn oil and animal fat.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Harold S. Weiss for his generous assistance and critical interest in this study. Acknowledgment is also made for material assistance from the Central Jersey Farmers Cooperative Association Inc., Hightstown, New Jersey; Staley Manufacturing Company, Decatur, Illinois and Dow Chemical Company, Midland, Michigan.

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NEWS AND NOTES

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WATT PUBLISHING NOTES

A. Gordruk has resigned as Editor of *Turkey World* and *Broiler Growing*, publications of the Watt Publishing Co., Mount Morris, Illinois, to become General Sales Manager of Nicholas Turkey Breeding Farms, Sonoma, California.

He graduated in 1941 from the College of Agriculture, Rutgers University, and after three and a half years in the Army, became an Associate County Agent in Massachusetts. In 1948 he joined the staff

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Effect of stearic acid and cholesterol on biosynthesis of sterol by the chicken liver[1]

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LIN, T. M., ESKO KARVINEN AND A. C. IVY. *Effect of stearic acid and cholesterol on biosynthesis of sterol by the chicken liver.* Am. J. Physiol. 198(1): 29–32. 1960.—The liver of the chicken synthesizes cholesterol from C^{14}-1-acetate. Stearic acid fed to chickens at a 10% level by weight in the diet was associated with a significant increase in the rate of hepatic cholesterol synthesis above that of the control group. Neither our observations nor those in the literature on other species reveal whether the observed augmentation was due to the saturation of the stearic acid. The feeding of cholesterol at a level of 2.5% by weight of the diet decidedly inhibited the hepatic synthesis of cholesterol whether 10% stearic acid was present in (76% inhibition) or absent from the diet (78% inhibition). A relatively large (23%) hepatic synthesis of cholesterol occurred when the diet contained more cholesterol than could be absorbed under the conditions of this experiment.

ALTHOUGH THE CHICKEN HAS BEEN USED rather extensively for studies on the production of atheromatosis, no studies appear to have been made on the synthesis of cholesterol by their liver (1–14 and unpublished data).

It is known that cholesterol feeding in the dog, rat, rabbit and monkey inhibits hepatic synthesis, but not intestinal synthesis to a significant extent (1–13 and unpublished data). The effect of cholesterol feeding on hepatic synthesis in human beings requires further study (11, 13, 14). The effect of cholesterol feeding on hepatic synthesis in the chicken has apparently not been studied. The effect of feeding fat on the hepatic synthesis of cholesterol has been studied in rats (3, 5, 7–9 and unpublished data). But the effect of stearic acid has not been studied.

This study was undertaken to determine the effect of a maximum amount of dietary cholesterol and of stearic acid on the hepatic synthesis of cholesterol.

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METHODS

One-week-old White Leghorn cockerels were used (Corn Belt Hatchery, Joliet, Illinois.) The basal diet (B.D.) consisted of a commercial mash containing 22% protein, 3% fat and 0.05% sterol, of which 5 mg was cholesterol, the remainder being phytosterol. *Group I* chicks received the B.D. only; *group II*, the B.D. plus 10% stearic acid (m.p. 69°C); *group III*, the B.D. plus 1.0% cholesterol for 10 weeks and 2.5% cholesterol for 6 weeks; *group IV*, the same as *group III* plus 10% stearic acid. The chicks were fed these diets for a total of 16 weeks ad libitum. They were then given 10 μc of C^{14}-1-acetate intraperitoneally 3 hours before they were killed. The chickens were bled well and the liver was immediately excised and weighed. It was then dried and pulverized and an aliquot was extracted with ether for 48 hours in a Soxhlet apparatus. After evaporating the ether, the residue was weighed for total lipids. Saponification was carried out with 10% alcoholic KOH and the nonsaponifiable material (UM) was extracted with ether after neutralization and weighed. Digitonin precipitable sterol was separated and weighed. Cholesterol in the serum and the feces was determined by the Sperry-Webb method (15). The material was prepared for counting by the procedures described by Tompkins *et al.* (5) and Gould *et al.* (6).

RESULTS

The essential results are summarized in table 1. The data on the fecal elimination of sterol are not provided. The chickens in *group II* eliminated more sterol than those in *group I*, but the increase was not statistically significant. The same was true for *groups III* and *IV*.

Effect of dietary cholesterol on hepatic synthesis of cholesterol. The hepatic synthesis of cholesterol on the basal diet is represented by 88 ± 15 c.p.m. (table 1, *group I*). When 2.5% cholesterol was fed, the synthesis of cholesterol was represented by 19 ± 39 c.p.m. (table 1, *group III*), which is a significant reduction amounting to 78%.

Effect of dietary stearic acid on hepatic synthesis of cholesterol. When stearic acid (10%) was fed the synthesis was

TABLE 1. *Effect of Dietary Cholesterol and Stearic Acid on Biosynthesis of Sterol From C^{14}-1-Acetate by the Chicken Liver*

Group	Diet	No. Chix	B. Wt.	Av. Serum Chol.	Liver (Average/Liver)							
					Liver wt.	B. wt./liver wt. ratio	Dry Wt.	Digitonide sterol				
								Total in liver	Wet liver	Dry liver	Total	Der. inc.
				mg %	gm		gm	mg	mg/gm	mg/gm	c.p.m./liver*	%
I	B.D.	4	1202	131	19.4	62	5.8	184	9.5	31.7	88 ± 15†	
II	B.D. + 10% stearic acid	5	960	182	16.1	63	4.8	150	9.3	31.2	208 ± 67†	+136
III	B.D. + 2.5% cholesterol	4	1105	1656	21.4	52	7.0	723	33.8	102.7	19 ± 39†	−78
IV	B.D. + 2.5% cholesterol + 10% stearic	4	1105	1277	24.2	45	7.7	850	35.1	109.7	21 ± 30†	−76

* c.p.m. = counts per minute. † Significance *I* vs. *II* = $t_{(7)} = 3.67 = P < .1$; *I* vs. *III* = $t_{(4)} = 3.92 = P < .05$; *II* vs. *IV* = $t_{(7)} = 5.37 = P < .01$.

decidedly increased from the control of 88 ± 15 c.p.m. to 208 ± 67, an increase of 136%. It should be noted (table 1, *group IV*) that the feeding of cholesterol completely annulled the augmenting effect of stearic acid feeding on cholesterol synthesis.

It should be emphasized that a considerable (23%) hepatic synthesis of cholesterol still occurred when the diet contained much cholesterol, in fact, more than could be absorbed under the dietary conditions. It has been found (manuscript in preparation) that the average intestinal capacity to absorb cholesterol in 1900 gm chickens was 210 mg at an intake of approximately 900 mg daily with a 1.2% concentration of cholesterol in the diet. And, in this study the cholesterol concentration in the diet was 2.5%.

DISCUSSION

Effect of feeding stearic acid. The factor or factors concerned in the increased hepatic synthesis of cholesterol associated with feeding stearic acid is uncertain. Since there is no literature on the chicken, our observations will be interpreted in relation to the literature on the rat.

Partial caloric undernutrition may be a factor because the chickens which received the stearic acid (10%) did not grow as well as the controls (table 1, *group I* and *II*). In the rat stearic acid is only 20% utilized (19, 20, 27) and in the chickens receiving stearic acid the stools were bulky and contained an increased amount of digitonin-precipitable sterol. So, caloric undernutrition may have been concerned in stimulating hepatic synthesis, although there was no other evidence of undernutrition. However, in the rat fasting is reported to decrease the hepatic synthesis of cholesterol; 50% after 48 hours, and 66% after 72 hours of fasting (16). We have also found that fasting in the rat decreases the hepatic synthesis of cholesterol from C^{14}-1-acetate (unpublished observations). Thus, if the chicken is like the rat in this regard partial caloric undernutrition was not involved.

A decrease in cholesterol absorption may have been a factor. In the rat it has been found that feeding a very low fat and cholesterol diet increased the hepatic concentration of cholesterol in the esterified form, though the serum cholesterol increased (17). When the absorbed cholesterol is diverted to the outside via a thoracic duct fistula, the hepatic synthesis of cholesterol is increased (18). When stearic acid (10%) was fed to rats, the excretion of endogenous and exogenous cholesterol was increased (19, 20). Since the concentration of sterol in the diet we fed was 0.05%, some of this may not have been absorbed, and cholesterol synthesis may have been indirectly stimulated. However, the fecal analysis showed only a slight increase in digitonin precipitable sterol. Since there was so little sterol in the diet, the increase most likely came from the endogenous sterol pool.

The literature on the rat, there being none on the chicken, is not entirely clear regarding the effect of the amount and type of fat on the cholesterol content of the liver. A diet very low in fat and cholesterol was associated with an increase in cholesterol ester in the liver (17). An increase in dietary fat from 3.5 to 25% by weight was associated in rats with a 29% increase in serum cholesterol when lard and oleic acid were fed, and a 33% increase when 2% cholesterol was added to these diets (21). When stearic acid was added to the diet containing 2% cholesterol by weight, the serum cholesterol did not increase as it did with lard and oleic acid (21). Later it was found that stearic acid interferes with the absorption of cholesterol (19). An increased accumulation of esterified cholesterol in the livers of rats fed sunflower oil (25%) (22), soya bean oil (22%) (23), corn oil (20%) (24), and linoleic acid (20%) (24), along with a reduction in serum cholesterol, has been observed. But this did not occur with coconut oil (20–22%) (23, 24). But, when lipids of different degrees of saturation, as determined by the iodine number, were fed at a 5% level, instead of the 20% level, neither the hepatic nor serum cholesterol concentration was affected (25). Hepatic cholesterol was correlated with the degree of unsaturation only when cholesterol was fed with the fat (25). Thus, it would appear that either a very low fat and cholesterol intake or a high fat intake with moderate cholesterol intake increases the cholesterol ester concentration of the liver of the rat. This particular factor will have to be studied specifically in the chicken by using synthetic diets.

TABLE 2. *Depression of Hepatic Synthesis by Cholesterol Feeding in Different Species*

Species	Type of Exp.	B. Wt.	Cholesterol Fed*			Decrease in Synthesis, %	Ref. No.
			Amt., mg/kg/day	Length of period of adminis.	Total dose		
Dog	*In vivo*	2 kg	700	8 da.	7 gm	93	1, 2, 4
Dog	*In vivo*	10 kg		1 dose 11 da.	16 gm	99	1, 6
Dog	*In vitro* *In vivo* Half-life	2.1	90	6 wk. ?	1.88 gm/da.	0 (?)	12
Rat	*In vivo*	230 gm	390	21 da.	2.1 gm	96	(Unpublished observations)
Rat	*In vivo*			1 da. 1 wk. 2 mo.	2% chol. + 5% cot. oil	99	9
Rat	*In vivo*			8 da.	9% chol. + 30% cot. oil	95	3
Rat	*In vitro*			8 da. 7 da.	5% chol. 0.5% chol. } cot. oil	98	5
Rat	*In vitro*			2 da.	1 mg/gm b. wt/da.	97	7
Rat	*In vitro*				1% chol.	93	8
Rabbit†	*In vitro*			6 wk.		Depressed markedly	1, 2
Monkey	*In vivo*			1 dose 4–6 wk.	Chol. and cream	Same as dog	10
Man‡	*In vivo*			‡	‡	Slight depression	11, 13
Chicken	*In vivo*	1–1.2 kg		1% 10 wk. 2.5% 6 wk.		77	This study

Rats and rabbits have faster rate of intestinal synthesis than of hepatic (14). * Cholesterol fed at the level of the capacity of the intestine to absorb it, or 90 mg/day. † Synthesis in liver proceeds slowly in rabbits (14) and was "depressed to a few per cent of that in the controls." ‡ That evidence is equivocal is indicated in ref. (14).

especially since the amount of cholesterol or sterol in the diet may determine the effect of a saturated or unsaturated fat.

In regard to the effect of fat feeding on hepatic cholesterol synthesis in the rat, it was found that feeding corn oil at a level of 20% by weight in the diet (85% of calories) for 2 or 3 months, not only increased the fat and cholesterol content of the liver but also decreased the capacity of the liver to synthesize fatty acids from C^{14}-1-acetate and increased the rate of hepatic synthesis (26). The feeding of corn oil (20%) and linoleic acid (20%) increased the hepatic concentration of cholesterol as the ester and the rate of synthesis of cholesterol from C^{14}-1-acetate; coconut oil (20%) did not (24). The diets used in both these studies were free of or very low in cholesterol, and high in fat. In the case of corn oil, the increased hepatic synthesis could be due to a lack of dietary cholesterol and a high fat diet, or both. Since increased synthesis did not occur when coconut oil was fed, the increased synthesis was specific for the type of fat fed. Intestinal absorption is probably not involved since it would have to be assumed that coconut oil increases the absorption of endogenous cholesterol enough to depress synthesis.

On the basis of the literature on the rat we are inclined to interpret our observations as indicating that depletion of body sterol by stearic acid feeding was compensated by an increased biosynthesis of sterol. This synthesis was excessive since the rise in the serum level of cholesterol after feeding stearic acid which decreased the absorption of sterol was statistically significant in this study and in another study (27). However, in view of the literature on the rat, the possibility that the amount and type of fat fed to the chicken has an effect in addition to its effect on the absorption of cholesterol has to be studied further.

Effect of feeding cholesterol. The feeding of 2.5% cholesterol decidedly depressed the hepatic synthesis of cholesterol, as has been observed in other species, and completely annulled the cholesterogenic effect of stearic acid feeding on the liver.

According to the literature (as summarized for various species in table 2) the hepatic synthesis of cholesterol is depressed 95% or more by feeding cholesterol to the dog (1, 2, 4, 6, 12), rat (3, 5, 7–9 and unpublished observations) rabbit (1, 2) and monkey (10). In human beings (11, 13) only a slight depression has been observed, but the observations may be equivocal because patients were used under conditions difficult to control (14). From the data available on other species and our data on the chicken, it appears that the chicken shows the least depression of hepatic cholesterol synthesis among the species studied, with the possible exception of the rabbit. Our data on the rat (unpublished), which showed 96% inhibition of hepatic synthesis by dietary cholesterol, checks with the literature (3, 5, 7–9), and is significantly greater than that (77%) which we observed in the chicken. Actual figures on the rabbit have not been found in the literature.

In the rat the assay on the liver was started by us (unpublished data) 2 hours after injecting the C^{14}-1-acetate; in the chicken the period was 3 hours; and, in the rabbit (1, 2) the period is not given. The length of this period is important in making comparisons because the rate at which the synthesis from C^{14}-1-acetate occurs may be slower in the chicken and reach a peak at 4 or 5 hours. The newly synthesized cholesterol reaches a peak in the plasma of a dog in about 1 hour and in man in about 4 hours (14). The rate at which hepatic synthesis is inhibited by the cholesterol fed may also vary among the species (1, 2, 14). This requires further study because the rate of inhibition of hepatic synthesis may be important in the determination of species susceptibility to atheromatosis. That it is not a decisive factor is indicated by the observation that in the rat (a resistant species) three to five times as much synthesized cholesterol is found in extrahepatic tissues as in the liver (14), and extrahepatic synthesis is reported not to be inhibited by cholesterol feeding to the extent that hepatic synthesis is (14).

Since the average capacity of the intestine of 2100–2200 cockerels to absorb cholesterol is 210 mg/day on a diet containing 1% cholesterol and 3% cottonseed oil by weight (manuscript in preparation), and since the diet fed in this study contained 2.5% cholesterol, the average 77% inhibition of hepatic cholesterol synthesis occurred in the presence of a close to the maximal absorption of cholesterol. If the chicken is like the rat, the cholesterol would have to be fed with 5 or 10% oleic acid to obtain a value for the maximal capacity of the chicken intestine to absorb cholesterol (28, 29).

The recovery of radioactivity in the cholesterol fraction depends on the rate of synthesis of cholesterol from acetate and on the specific activity of the acetate in the acetate precursor pool. In the preceding discussion it has been assumed that the second factor is not affected by the various diets used.

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Influence of Dietary Saturated and Unsaturated Fats on Blood Coagulation In Miniature Pigs.*† (28653)

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Following the observations of Keys and coworkers(1,2) that the fat content of a diet bears a direct relationship to the plasma cholesterol level of the population and to the incidence of coronary heart disease, it was shown by other investigators(3,4) that unsaturated fats decreased the plasma cholesterol level and that saturated fats increased it. Whether saturated and unsaturated types of dietary lipids can affect coagulability of blood, as well as possibly contributing to the development of atherosclerosis, is not known. Numerous studies have attempted to ascertain whether, and to what extent, dietary fats bring about changes in blood coagulation and fibrinolytic systems. Both animal and vegetable fats produced the same shortening of the recalcification time of plasma of patients, 3 and 4 hours after ingestion of fat(5). Keys *et al*(6) found that the silicone clotting time of blood was much shorter on a butterfat or coconut oil diet than on a corn oil diet. Using pigs as experimental animals, Rowsell *et al*(7) suggested that feeding butterfat increases both the incidence and severity of aortic lesions, perhaps due to alterations in the blood clotting mechanisms.

At The Hormel Institute, miniature pigs are being used in studies of the fundamental causes of atherosclerosis, including the influence of various dietary fats on blood coagulation. This paper presents some of the differences observed in the coagulability of blood on miniature pigs maintained for a

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† These results are part of an experiment designed and supervised by Dr. Eldon G. Hill, the results of which will be reported later. Our thanks are due to M. C. L. Silberwick, for technical assistance in this work.

TABLE I. Composition of Diets.

Ingredient	Group I	Group II
	(%)	
Corn	33	33
Oat groats	30	30
Soybean oil meal (solvent ext'd)	10	10
Dry skim milk	5	5
Meat and bone scraps	5	5
Minerals*	1.05	1.05
Vitamin mix†	.85	.85
Cholesterol	.5	.5
Beef tallow	15.0	—
Safflower oil	—	15.0
	100.4	100.4

* Minerals added to ration: 0.5% dicalcium phosphate, 0.5% iodized salt, 0.05% Hi-Zinc trace-mineral mix for swine (Calcium Carbonate Co.).

† Vitamins added to ration: 0.2% Vit. B mix (2-4-9-10), 0.05% dry stabilized Vit. A palmitate and Vit. D_2 mix (as Quaknex IV, Nopco, 10,000 units of A and 1250 units of D_2 per g); 0.1% dry stabilized Vit. E acetate (20,000 units/lb); 0.5% Vit. B_{12} and antibiotic mix (Aurofac).

year on diets containing beef tallow and safflower seed oil.

Materials and methods. Twelve weanling miniature pigs were fed a low fat basal diet for 3 weeks and then were divided into 2 groups. One group was supplemented with 0.5% cholesterol and 15% beef tallow and the other with the same amount of cholesterol and 15% safflower oil. The composition of the diets is shown in Table I. Diets were fed on an equal basis of calories/kg of body weight. The animals were equilibrated with these diets for one year. The average weight of the animals of the tallow group was 310 lb. and that of the safflower group, 260 lb. The general appearance of all the animals was very good. Fasting blood samples were taken in the morning. Lipemic blood was taken 3 hours after feeding following an overnight fast. Samples of blood were obtained from the anterior vena cava with siliconized needles and syringes by the 2-syringe technique.

Whole-blood clotting time was measured by a modified 2-tube Lee-White method (2 ml whole blood per tube). A stop watch was started at the instant the needle was withdrawn from the animal. The first tube was tilted at one-minute intervals until a firm clot was obtained. The second tube which was kept undisturbed was thereafter tilted at 30-second intervals until clotting occurred. The clotting time of the second tube was taken as the whole-blood clotting time.

The silicone clotting time was measured in a single siliconized tube containing 2 ml of blood. The tube was tilted at 30-second intervals after clotting had occurred in unsiliconized tubes, and the clotting time recorded.

Plasma was prepared by mixing blood with a 20% sodium citrate solution (0.2 ml/10 ml blood) and centrifuging at 2500 RPM for 15 minutes. Recalcified plasma clotting time (R. P. C. T)(8), prothrombin time (one-stage, using simplastin‡)(9), stypven§ time (10), and plasma optical density at 640 mμ were determined.

Results. Table II shows the mean whole-blood clotting times in glass and silicone coated tubes. The optical density of the fasting plasma obtained from both groups of pigs was almost identical and indicative of the absence of hyperlipemia. Yet clotting time of the tallow group was shorter than that of the safflower group both in the silicone tube ($P < 0.05$) and in the glass tube ($P < 0.025$). In the lipemic state, *i.e.*, 3 hours after feeding, the shortening of the clotting time in the tallow group was much more pronounced ($P < 0.001$). However, the plasma turbidity of the tallow group was much higher than that of the safflower group. Consequently, the shortening of the clotting time of the tallow group might be related in part to the degree of lipemia.

The results of the recalcified plasma clotting times (R. P. C. T.), prothrombin times and stypven times of both groups in the fasting and lipemic states are shown in Table II. In the fasting state, the R. P. C. T. of the tallow group was much shorter than that of the safflower group, whereas there was no significant difference in prothrombin and stypven times between the two groups. In the lipemic state the R. P. C. T. of the tallow group was much shorter than that of the safflower group. Although there was no difference in prothrombin time between the two groups, the stypven time was shorter

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TABLE II. Blood Coagulation of Miniature Pigs Equilibrated for One Year on Diets Containing Beef-Tallow or Safflower Oil.

Group	Fasting state A Safflower oil	Fasting state B Tallow	Significance of differences: A – B	Lipemic state† C Safflower oil	Lipemic state† D Tallow	Significance of differences: C – D
Whole blood clotting time (min):						
Silicone tube	20.1 ± 2.44*	14.9 ± 4.9	P <.05	13.6 ± 2.1	4.6 ± 2.6	P <.001
Glass tube	11.5 ± .75	9.3 ± 1.6	P <.025	10.25 ± 1.3	4.2 ± 2.4	P <.001
Plasma O.D.	.12 ± .02	.122 ± .03		.218 ± .09	.406 ± .19	
R.P.C.T. (sec)	78.4 ± 14.8	53.8 ± 5.6	P <.005	60.8 ± 11.2	35.8 ± 1.9	P <.001
Prothrombin time (quick) (sec)	14.9 ± 1.3	15.2 ± .9	N.S.‡	14.2 ± 1.2	13.5 ± 1.6	N.S.
Stypven time (sec)	21.8 ± 2.9	20.5 ± 2.1	"	19.3 ± 2.2	15.2 ± 1.2	P <.01

* In all columns, refers to mean ± standard deviation.
† 3 hr after feeding following overnight fast.
‡ Not significant.

in the animals receiving tallow. Again, it should be pointed out that both fats were capable of shortening the R. P. C. T. and stypven time 3 hours after ingestion of the respective fatty meals, and the reduction of these values brought about by tallow was much more than that induced by safflower.

Discussion. The influence of various fat-containing diets on blood coagulation has been studied by several investigators to determine the possible relationship of diet to thrombosis. Most of these studies have been concerned with the effects on coagulation of a single fatty meal or very short term feeding. The present work is concerned with measurements of coagulation times of blood of miniature pigs maintained for one year on diets containing 2 different fats: 1) beef tallow, a saturated type of fat, and 2) safflower oil, an unsaturated type of fat. The experimental diets were designed to approximate the normal human diet in that about 40% of the calories were fat calories. The animals were equilibrated on these diets for one year so that lipid distribution in the blood of these animals would reflect, to some extent, the nature of the dietary lipids. Lipid analyses will be reported later.

Even though no hyperlipemic condition was observed in the fasting state, the mean whole-blood clotting time of the tallow group was shorter than that of the safflower group, in both glass and silicone tubes. In the lipemic state, both fats shortened the whole-blood clotting times in glass and silicone tubes; the effect produced by tallow was greater than that produced by safflower oil. This could be ascribed to the higher degree of lipemia observed in the plasma of the tallow group. However, it is doubtful whether the same degree of lipemia would produce identical clotting times in both groups. Correlation of clotting time with degree of lipemia in both groups needs further study.

The mean R. P. C. T. of the tallow group is also shorter in fasting and lipemic states. There is no difference in the stypven time of both groups in the fasting state, whereas it is shortened in both groups 3 hours after feeding their respective diets. In the lipemic state, the stypven time of the tallow group was much lower than that of the safflower oil group ($P < 0.01$). Perhaps this difference is the result of the difference in the plasma turbidity levels of the two groups. If so, this is consistent with the view that the stypven time test is a rough index of lipemia(11). There was no difference in prothrombin time between the two groups either in the fasting state or 3 hours after ingestion of their fatty meal.

Prothrombin time is not affected by lipemia, nor is it useful to detect differences in coagulability brought about by saturated and unsaturated fats. Although the stypven time is affected by lipemia, its usefulness for the latter purpose still remains to be demonstrated. The R. P. C. T. on the other hand is

significantly different in the two groups, that of the tallow group being much shorter than the safflower oil group. The mechanism by which dietary fats influence clotting times including stypven and recalcification times is not known. Studies on the influence of dietary fats on various clotting factors and on rate of thromboplastin generation in miniature pigs are in progress.

Summary. Miniature pigs maintained for one year on diets containing 15% of either beef tallow or safflower oil did not exhibit lipemia in the fasting state, yet the clotting time was much shorter in the tallow group than in the safflower group. The clotting time was shortened 3 hours after ingestion of either fat, but the effect produced by the saturated fat was much more pronounced than that produced by the unsaturated fat. The recalcified plasma clotting time was also a satisfactory measure of the increase in coagulability brought about by dietary fats, and served to detect differences in coagulability brought about by saturated and unsaturated fats.

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ADENOSINE TRIPHOSPHATASE AND MORPHOLOGICAL INTEGRITY OF MITOCHONDRIA*

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Fresh rat liver mitochondria prepared in 0.25 *M* sucrose have little or no ATPase*** activity[1-4]. However, such agents as dinitrophenol and gramicidin are capable of stimulating ATP hydrolysis by these same particles. In contrast, aging of the mitochondria at 37° appeared to be necessary to demonstrate enhancement of ATPase by the thyroid hormones[5,6]. It was suggested[6] that the aging process permitted better access of the activator to a receptor site on the mitochondria. Since these earlier studies on ATPase were made at 23°, it was thought possible for the thyroid hormones to activate fresh mitochondrial ATPase if assayed at a higher temperature. Comparisons of ATPase activities under the influence of the thyroid hormones were made at 23°, 30°, and 37°. As shown in Fig. 1, both thyroxine and triiodothyronine (TRIT) stimulated ATPase activity in fresh mitochondria at 30° and 37°, but not at 23°.

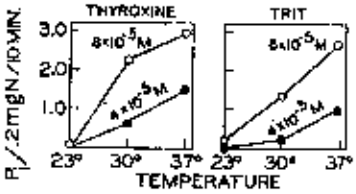

Fig. 1. Effect of temperature on the stimulation of fresh mitochondrial ATPase by the thyroid hormones. The reaction mixtures contained 6 μmoles of ATP, the appropriate aliquot of thyroxine or triiodothyronine, mitochondria from 60 mg wet liver, and isotonic KCl to a final volume of 1.0 ml. After 10 minutes incubation the reaction was stopped by addition of cold 10% TCA and inorganic phosphate was determined on the supernatant after centrifugation[10]. The figures have been corrected for the appropriate controls run in the absence of thyroid hormone.

While these studies were in progress, TAPLEY *et al.*[7] demonstrated that thyroxine and related compounds cause swelling of mitochondria. This suggested that the apparent stimulation of mitochondrial ATPase elicited by some agents may be a reflection of their effect on mitochondrial structure, or, that the enhanced ATPase activity causes loss of mitochondrial integrity[8]. An attempt to correlate these activities was, therefore, made.

METHODS

Mitochondria were isolated from livers of normal rats in 0.25 *M* sucrose[8]. Each ml of the final mitochondrial suspension represented 200 mg of wet liver. Duplicate reaction mixtures were set up to be incubated at 23° and 37° respectively. Each tube contained 1.0 ml of 0.06 *M* ATP.

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*** The following abbreviations will be used: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; DNP, 2,4-dinitrophenol; TCA, trichloroacetic acid; TRIT, triiodothyronine; P_i, inorganic phosphate.

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the appropriate aliquot of the compound being tested, 3.0 ml of the mitochondrial suspension and isotonic KCl to a final volume of 10 ml. At intervals of 0, 5, 10, and 15 minutes, two 1-ml aliquots were removed from the reaction mixtures. Inorganic phosphate was determined on the trichloroacetic acid supernatant of one aliquot[10]; ATPase activity is expressed as μmoles P_i released per mg N per given time interval. The other 1-ml aliquot was pipetted directly into 9 ml of isotonic sucrose and swelling was immediately determined as the decrease in density at 515 mμ measured in the Evelyn colorimeter. The data in Table I have been corrected for the controls which were run in each experiment in the absence of any agent. The average control values are shown in the first line of Table I. All compounds were tested at several concentrations in at least two separate experiments. The results summarized in Table I are the averages of the data obtained at that concentration of each compound which gave maximal ATPase stimulation in this assay.

TABLE I

COMPARISON OF MITOCHONDRIAL ATPASE AND STRUCTURAL CHANGES

	Compound	23°		37°	
		μmoles P_i/0.2 mg N	"swelling"	μmoles P_i/0.2 mg N	"swelling"
	None	0.05	—	0.45	—
1 γ/ml	gramicidin S	2.42	—	3.93	—
$3 \cdot 10^{-5}$ M	dinitrophenol	2.10	—	3.75	+
10 γ/ml	antimycin A	1.08	—	2.38	—
$6 \cdot 10^{-5}$ M	butyl-3,5-diiodo-4-hydroxy benzoate	1.66	—	3.66	+
1.5 mg/ml	saponin	1.91	+	3.72	+ + —
50 γ/ml	desoxycholate	1.80	+	3.32	+ + —
$6 \cdot 10^{-5}$ M	triiodothyroacetic	1.68	+	3.12	+ + —
$8 \cdot 10^{-5}$ M	thyroxine	0.10	—	2.22	+ + +
$8 \cdot 10^{-5}$ M	triiodothyronine	0.17	—	2.17	+ + +
100 γ/ml	stearic acid	0.23	—	1.45	+ + +
$3 \cdot 10^{-3}$ M	DPNH	0.08	—	0.95	+ +
$3 \cdot 10^{-3}$ M	DPN	0.02	—	0.21	—
$3 \cdot 10^{-3}$ M	cysteine	0.13	—	0.60	—
3%	ethanol	0.10	—	0.46	—
60%	water (hypotonic)	0.02	—	0.55	—

The reaction medium is described in the text. Figures represent the averages of two experiments for each compound tested and have been corrected for the control. The degree of swelling is based on change in density as read at E_{515}. + represents change of 0.02–0.05; ++ 0.05–0.1; +++ 0.10–0.20 under the conditions of this assay.

Reaction time = 10 minutes.

RESULTS AND DISCUSSION

Gramicidin, antimycin A, and butyl-3,5-diiodo-4-hydroxybenzoate react like dinitrophenol in that they stimulate the ATPase of fresh mitochondria without causing a pronounced swelling of these particles[7,11]. Desoxycholate, saponin, and triiodothyroacetic acid produce swelling or rupturing of mitochondria and activate ATPase both at 23° and 37°. Triiodothyronine, thyroxine, stearic acid, and DPNH cause loss of mitochondrial integrity and enhance ATPase at 37° but not at 23°. Cysteine, water (or hypotonicity), ethanol, and DPN are relatively inactive under these conditions at the particular concentrations reported here. The great differences in concentration necessary for activation of ATPase by saponin and desoxycholic acid have been described previously[12].

The time course for the activation of ATPase and change in mitochondrial density

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is plotted in Fig. 2. Results with dinitrophenol, triiodothyronine, and desoxycholate are shown since they are typical of the three types of change brought about by the compounds tested in this study. It is interesting to note that there is some swelling of mitochondria with dinitrophenol at 37°. This may be due to the extensive loss of ATP under these conditions for it has been reported[11] that dinitrophenol *per se* does not affect mitochondrial structure other than to prevent the swelling caused by thyroxine. The work of RAAFLAUB has shown loss of ATP to produce swelling of mitochondria[8].

In the present studies there has been no direct attempt to determine whether ATP breakdown precedes mitochondrial swelling or vice versa. However, it would appear from the results of this study and those of others[8,11] that (1) both conditions may occur simultaneously, as in the presence of desoxycholate, saponin, or triiodothyroacetic acid; (2) activation of ATPase may precede swelling, as in the presence of DNP, gramicidin, antimycin A, and butyl-3,5-diiodo-4-hydroxybenzoate; or (3) swelling may precede ATPase activation, as in the presence of thyroxine, triiodothyronine, and stearic acid.

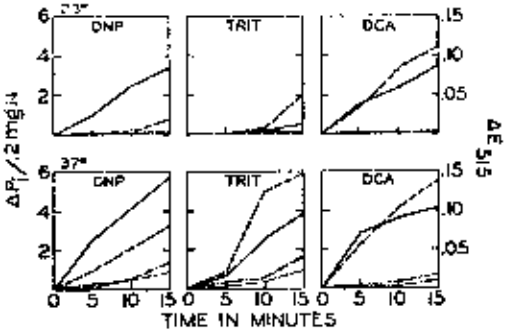

Fig. 2. Effect of time and temperature on the ATPase and swelling of mitochondria treated with dinitrophenol (DNP), triiodothyronine (TRIT), and desoxycholate (DCA). The experimental procedure is given in the text. The concentrations of DNP, TRIT, and DCA are the same as those given in Table I. —— ATPase activity in the presence of DNP, TRIT, or DCA; —·—·— ATPase activity in the control; ----- swelling in the presence of DNP, TRIT or DCA; ····· swelling in the control.

It should be emphasized that hydrolysis of ATP is the result of activity of a number of different enzymes[13,14] some of which probably function as transphosphorylases before structural damage to the mitochondria permits them to transfer to water[13]. The complications involved in interpretation of results seem to render premature any judgement as to the mechanism by which the thyroid hormones affect ATP hydrolysis. Evidence for these complications is presented in Table II. Thyroxine enhanced ATP hydrolysis in the presence of concentrations of Ca^{++} which did not maximally stimulate ATPase. However, at higher concentrations of Ca^{++}, thyroxine had no effect.

TABLE II

INFLUENCE OF THYROXINE ON MITOCHONDRIAL ATPASE IN THE PRESENCE OF CALCIUM

Additions	*μmoles P_i per 0.2 mg N*	
		1.4×10^{-5} M thyroxine
None	.04	.08
10^{-2} *M* Ca^{++}	1.06	2.14
$3 \cdot 10^{-3}$ *M* Ca^{++}	1.60	1.56

Reaction medium contained 6 μmoles of ATP, mitochondria from 30 mg fresh liver, the additions above and isotonic KCl to a final volume of 1 ml. Incubation at 23° for 10 minutes. Reaction stopped with TCA and inorganic phosphate determined as in the text.

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In other experiments isotonic sucrose replaced isotonic KCl in the reaction medium. It was found that, in general, the two types of media gave similar results. It was observed that with very dilute suspensions of mitochondria incubated for relatively long time intervals, cysteine caused a striking disappearance of mitochondrial structure. In simple swelling experiments we could confirm TAPLEY's[11] observation that both versene and ATP plus Mg^{++} completely prevent the thyroxine induced swelling of mitochondria. ATP alone, Mg^{++} alone, and DNP only partially prevented this effect.

KLEMPERER[15] has recently reported that mitochondria prepared in KCl show considerable ATPase activity in the fresh state which is further enhanced by addition of DNP or thyroxine. However, DNP, but not thyroxine, can stimulate the ATPase of aged KCl-mitochondrial preparations. This appears to be a major difference in the KCl and sucrose mitochondrial preparations since the opposite has been observed with sucrose prepared mitochondria. AEBI AND ABELIN[16], BEYER *et al.*[17], and TAPLEY[11] have shown that mitochondria from thyrotoxic rat liver swell more rapidly than those from normal rat liver. This is in agreement with the earlier release of ATPase from thyrotoxic mitochondria during aging at 37°[18].

SUMMARY

Gramicidin, antimycin A, dinitrophenol, and butyl-3,5-diiodo-4-hydroxybenzoate stimulate mitochondrial ATPase at 23° without causing swelling of these particles. Desoxycholate, saponin, and triiodothyroacetic acid activate ATPase at 23° but also produce swelling or rupturing of the mitochondria. Triiodothyronine, thyroxine, stearic acid, and DPNH enhance mitochondrial ATPase and swelling at 37° but not at 23°.

At suboptimal levels of Ca^{++}, thyroxine further actives fresh mitochondrial ATPase but has no effect when the Ca^{++} concentration is optimal.

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Fat Studies in Poultry

6. UTILIZATION OF FATS OF DIFFERENT MELTING POINTS*

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DUCKWORTH, Naftalin and Dalgarno (1950) found that the difference in digestibility between low and high melting point fats reported for other animals exists also for the chick. Carver *et al.* (1955) and Sunde (1956) reported that hydrogenated fat is poorly utilized by the chick. The following experiments were conducted to study the nutritive value for the growing chick of fats of different saturation. The effect of dietary fat level and of feeding surface active agents on the utilization of fat has been studied. Since there is little data available regarding the form in which fatty acids are excreted by the chick, the ratio of split to unsplit fat excreted has been determined.

EXPERIMENTAL

Experiment 1. The composition of the rations used in the first experiment is shown in Table 1. The basal ration included 12 percent cerelose. In the rations in which fat was included, cerelose was replaced on a pound for pound basis with 3, 6, 9 and 12 percent of either hydrogenated animal fat (HAF) or corn oil. The melting point of the HAF used in both experiments was approximately 55°C. Each ration was fed to duplicate lots of 20 New Hampshire male chicks to 4 weeks of age. The average weights of the chicks at 4 weeks and the efficiency of feed utilization from 0-4 weeks are shown in Table 2.

The fat content of the wet feces from the chicks was determined by the method of Hoagland and Snider (1943). The procedure was modified slightly in that fatty acids were extracted with 4-50 ml. portions of petroleum ether.

The digestibility coefficients of the fat in the different rations are given in Table 2. The values represent an average of the results for three separate 24 hour periods on the 15th, 19th and 24th days of the experiment. The digestibility coefficients were not corrected for metabolic fat by itself but were calculated by correcting for the total fat (metabolic and undigested dietary) excreted by the chicks fed the basal ration.

Experiment 2. In Experiment 2, HAF was compared to beef tallow when fed in a chick ration at a level of 12 percent.

The effect of surface active agents on fat utilization was studied with 0.5 per-

TABLE 1.—*Composition of basal diets*

Ingredient	Experiment 1	Experiment 2
Ground yellow corn	22.0	25.25
Ground wheat	22.0	25.25
Cerelose	12.0	12.00
Soybean oil meal (solvent extracted)	26.00	13.00
Fish meal	10.0	13.00
Meat meal	—	5.00
Limestone	1.0	0.5
Bonemeal	1.5	0.5
Dehydrated cereal grass	2.5	2.5
Dried butyl fermentation solubles	2.0	1.0
Dried brewers' yeast	—	1.0
Iodized salt	0.5	0.5
Feeding oil 2250A (3001)	0.25	0.25
Choline Chloride (25%)	0.25	0.25
Manganese Sulphate	6 gm.	6 gm.
Protein (calculated)	25%	25%

* Carried out with the aid of a grant from the Canada National Research Council.

† Contribution Number 93.

TABLE 2.—*Summary of data in Experiment 1*

Fat supplement to basal diet	Average weight (grams) at 4 weeks	Feed conversion ratio	Digestibility coefficient of added fat
None	386	1.99	
3% HAF	409*	2.05	51.0
6% HAF	392	1.96	50.0
9% HAF	378	1.97	50.0
12% HAF	364*	2.06	44.2
3% corn oil	408*	2.00	84.7
6% corn oil	418**	1.81	92.3
9% corn oil	417**	1.79	90.8
12% corn oil	402	1.90	91.8

* Significantly different from average control weight at 5% level of significance.
** Significantly different from average control weight at 1% level of significance.
MSD at 5% level 19.
at 1% level 25.

cent lecithin and 0.1 percent Santomerse-80 respectively in the diets. The composition of the basal ration is shown in Table 1. Each ration was fed to 23–25 New Hampshire male chicks for 6 weeks. The average weights of the chicks and the efficiency of feed utilization for this period are summarized in Table 3.

The procedure of Van de Kamer *et al.* (1949) was followed in this experiment for the determination of fat in feces. In this method fatty acids present in soap or as free fatty acids are determined separately from fatty acids present in neutral fat. Determinations were made on the feces collected on the 26th, 27th and 28th days of the experiments. The

TABLE 4.—*Utilization of supplementary fat in Experiment 2*

Supplement to basal diet	Coefficient of Digestibility[1] %	Coefficient of Digestibility[2] %	Proportion of fat excreted as neutral fat %
12% HAF	73.5	23.1	34.5
12% HAF plus lecithin	68.3	23.3	41.3
12% HAF plus Santomerse-80	70.8	23.2	38.0
12% tallow	99.1	75.2	3.7
12% tallow plus lecithin	99.6	72.8	1.5
12% tallow plus Santomerse-80	99.9	72.5	0.3

[1] Calculated from the neutral fat content of the feces.
[2] Calculated from the total fatty acid content of the feces.

digestibility coefficients of the two fats with and without surface active agents were calculated on the basis of the neutral fat excreted as well as on the basis of the total fatty acids excreted.

The coefficients of digestibility of the fat supplements were calculated with correction for the fat excreted by the chicks fed the corresponding diet without added fat. When a surface active agent was included in the diet, the correction was made on the basis of the fat excreted by the chicks fed the basal diet supplemented with the surface active agent. The data regarding fat utilization are shown in Table 4.

RESULTS AND DISCUSSION

In Experiment 1 when 3, 6 and 9 percent of corn oil were added to the basal diet, growth of chicks was significantly faster. Twelve percent corn oil did not stimulate growth significantly. Feed ef-

TABLE 3.—*Average weights and feed efficiency of chicks at 6 weeks in Experiment 2*

Supplement to basal diet	None		Lecithin		Santomerse-80	
	Av. wt. (grams)	Feed conversion ratio	Av. wt. (grams)	Feed conversion ratio	Av. wt. (grams)	Feed conversion ratio
none	716	(2.32)	695	(2.34)	658	(2.41)
12% HAF	639	(2.55)	632	(2.54)	666	(2.40)
12% beef tallow	703	(2.20)	683	(2.20)	653	(2.22)

MSD at 5% level 55.

ficiency was improved by the addition of 6 or 9 percent of corn oil to the diet and to a lesser extent, by 12 percent of corn oil.

The addition of 3 percent of HAF to the basal diet stimulated growth, but 6 and 9 percent of HAF were without effect. When added at the 12 percent level HAF depressed growth. HAF did not affect feed efficiency although it was itself poorly utilized. There was no effect on feed utilization with any of the levels of HAF used.

HAF was only 50 percent utilized by the chick when fed at levels of 3, 6 or 9 percent and had an even lower coefficient of digestibility (44 percent) at the 12 percent level. The digestibility coefficient of the corn oil increased from approximately 85 percent when fed at the 3 percent level to 91–92 percent at dietary levels of 6, 9 and 12 percent.

Since the melting point of the HAF was approximately 55°C., the poor digestibility was not unexpected. Duckworth, Naftalin and Dalgarno (1950) found that the difference in digestibility between low and high melting point fats reported for other animals exists also for the chick. Carver *et al.* (1955) reported that "neither hydrogenated tallow nor hydrogenated tallow fatty acids are efficiently utilized as fat supplements to broiler feed." Sunde (1956) concluded that the chick does not utilize saturated long chain fatty acids.

The digestibility coefficient of the fat was influenced by the level at which it was fed. Corn oil was utilized equally well at levels of 6, 9 and 12 percent but not so completely when it comprised only 3 percent of the diet. HAF, on the other hand, was utilized to a similar extent whether fed at a level of 3, 6 or 9 percent of the diet, but was less well utilized when the level was raised to 12 percent. The drop in utilization of the HAF at this level was not reflected in the overall efficiency of feed utilization. These data do not agree with those on rats of Hoagland and Snider (1943) which indicated increased digestibilities for the least digestible fats when the intake was increased. They are in accord with those of Duckworth, Naftalin and Dalgarno (1950) however, which showed that the digestibility of mutton fat for the chick was depressed when the level was increased beyond 6 percent of the diet.

In Experiment 2 the addition of 12 percent of HAF to the basal diet depressed the rate of growth of chicks fed the diet. Feed efficiency, likewise, was adversely affected. The addition of 12 percent beef tallow, on the other hand, although not affecting growth rate, did improve the efficiency with which the ration was utilized. Supplementation of the rations with 0.5 percent of lecithin had no effect on either the rate of growth or the efficiency of feed utilization. Santomerse-80 depressed growth of chicks fed either the basal diet or the diet containing 12 percent tallow but had little or no effect on feed efficiency. The addition of Santomerse-80 to the diet containing 12 percent HAF did not depress growth and improved the efficiency with which the diet as a whole was utilized.

On the basis of total fatty acids excreted, the digestibility of the hydrogenated fat in this experiment was only 23 percent. The discrepancy in the values obtained for the digestibility of the HAF between the two experiments was most probably due to the fact that a different sample of HAF was tested in each experiment and also that there were slight differences in the form in which calcium was supplied in the diets. Cheng, Morehouse and Deuel (1949) observed that the dietary levels of calcium and mag-

nesium had a marked effect on the digestibility of hydrogenated fats. Fat digestibility was not affected by the addition of a surface-active agent to the diet.

Since the analytical procedure determined separately neutral fat and fatty acids, free and as soaps, it was possible to calculate the relative proportion of fat excreted in these forms. Following the same method of calculation as previously, the digestibility coefficients of HAF and tallow were also determined on the basis of the neutral fat excreted. From data shown in Table 3 it will be noted that when the digestibility coefficients were calculated in this way the data are more in accord with those in the literature. It is felt, however, that any method which does not take fecal soaps into consideration in the calculation of digestibility coefficients of fats for the chicken cannot be considered as valid. Crockett and Deuel (1947), studying fat digestibility in the rat, made similar observations on the discrepancy between digestibility coefficients calculated with and without allowance in the calculations for the soaps excreted.

In the case of the chicks fed tallow, only a small percentage (3.7 percent) of the fecal fat was in the form of neutral fat. The addition of lecithin to the diet reduced the percentage of tallow excreted as neutral fat to 1.5 percent. When Santomerse-80 was added to the diet with tallow, the amount of neutral fat excreted was scarcely more than was excreted by the chicks fed the basal diet with Santomerse-80 as supplement. When HAF was added as fat supplement, 34.5 percent of the fat in the feces was in the form of neutral fat. Presumably much of the neutral fat in the feces represented fat excreted unchanged from the form in which it was fed. Visual examination of the feces, in fact, showed small particles of the HAF which had not been completely broken down during passage through the digestive tract. The addition of lecithin to the diet containing HAF increased the proportion of neutral fat in the feces to 41.3 percent and Santomerse-80 increased it to 38.0 percent.

It has been indicated that, in the rat, the larger proportion of the fat excreted when saturated fats are fed is in the form of soap. Augur, Rollman and Deuel (1947) and Crockett and Deuel (1947) reported that, in rats, the highly saturated fats were not resistant to hydrolysis in the intestine but that the soaps formed from saturated fatty acids could not be absorbed. In the present experiment it will be seen that with both fats tested, the greater part of the excreted fat was in the form of soap and free fatty acid. In the feces from chicks fed HAF the proportion of split fat to unsplit fat was lower, however, than in the feces from chicks fed the less saturated tallow. This would indicate that in the chick the highly saturated fats are resistant to hydrolysis.

The effect of the surface-active compounds was interesting when considered in terms of the findings of other investigators. Deuel (1955) states that it would be unlikely to expect an improvement in fat digestibility by the addition of a surface active agent to a diet containing a fat already possessing a high coefficient of digestibility. In the present experiment there was no indication that lecithin or Santomerse-80 had any beneficial effect on the utilization of either fat tested. On the basis of the relative proportions of neutral fat and fatty acids (free and as soap) excreted when 0.5 percent lecithin was added to the diet supplemented with HAF, there was a shift towards a higher proportion of neutral fat in the feces. Santomerse-80 had a similar, though lesser, effect to lecithin. When the sur-

face-active agents were added to the diet supplemented with tallow, the action was in the reverse direction. The proportion of tallow excreted unhydrolyzed was very small in the case of the diet without surface-active agents and was reduced still further by either lecithin or Santomerse-80. With the latter compound, the amount of neutral fat excreted on the diet with tallow added was reduced to such a degree that the total neutral fat present in the feces was no more than was excreted on the corresponding basal diet without added tallow.

SUMMARY

Corn oil, tallow and hydrogenated animal fat were studied as supplements to chick rations.

Corn oil and tallow were well utilized by the chick. The coefficients of digestibility were not so high as those reported in the literature because fatty acids excreted in the form of soaps were taken into account in the present experiments. When fed at the 12 percent level the values obtained were approximately 90 percent for corn oil and 73 percent for tallow. Two samples of hydrogenated animal fat fed at the 12 percent level were utilized only to the extent of 44 and 23 percent respectively.

Although there were differences in the distribution of fatty acids in the feces between split and intact fat when lecithin or Santomerse-80 was added to rations supplemented with either tallow or hydrogenated animal fat, there was no appreciable improvement in the digestibility of either fat.

The greater part of fat excreted was in the form of soaps and free fatty acids. Chicks fed hydrogenated animal fat excreted a higher proportion of fat as triglyceride than did chicks fed the less saturated tallow. Highly saturated fats are evidently resistant to hydrolysis in the chick.

Fecal soaps must be considered in any estimation of digestibility of fat by the chicken.

ACKNOWLEDGMENT

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Pharmacological Effects of Fatty Acids, Triolein and Cottonseed Oil.

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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

[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 compositons 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	0.2 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 I – *into mice* only a transient depression of the respiration rate. It follows that the LD50 is higher than 15 g cottonseed oil per kilogram.

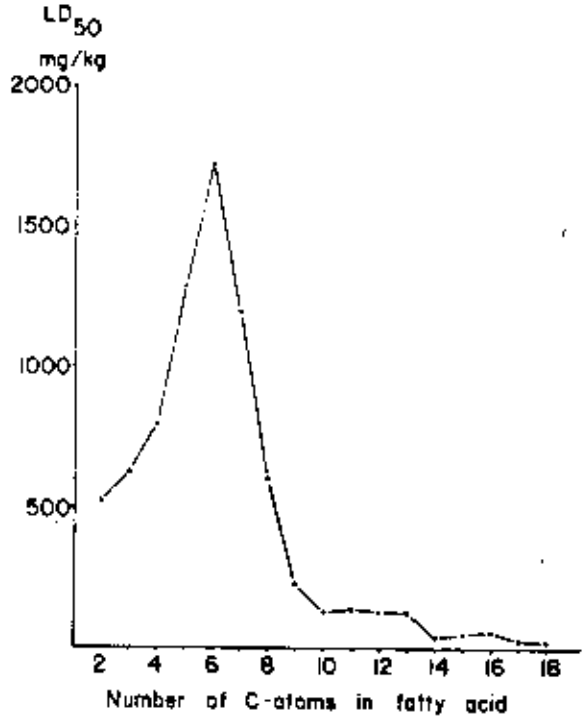

Fig. 1. *Toxicity of saturated fatty acids on intravenous injection into mice.* The abscissa indicates the number of carbon atoms in the fatty acid molecule; the ordinate, LD50 in milligrams per kilogram of body weight. The LD50 values in mg/kg, together with their standard errors, were: acetic acid 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 acid* 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.

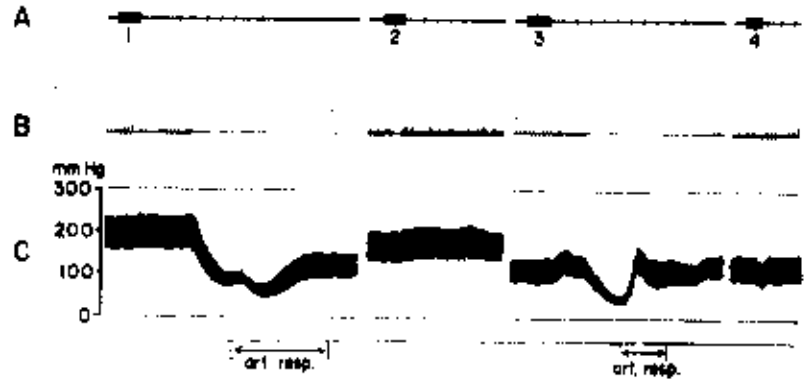

Fig. 2. *Influence of cottonseed oil emulsion on blood pressure and respiration.* Cat, 3.0 kg, under Dial R anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. "Art. resp." denotes artificial respiration. At 1, 2, 3 and 4, 0.7 ml/kg of 10% cottonseed 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

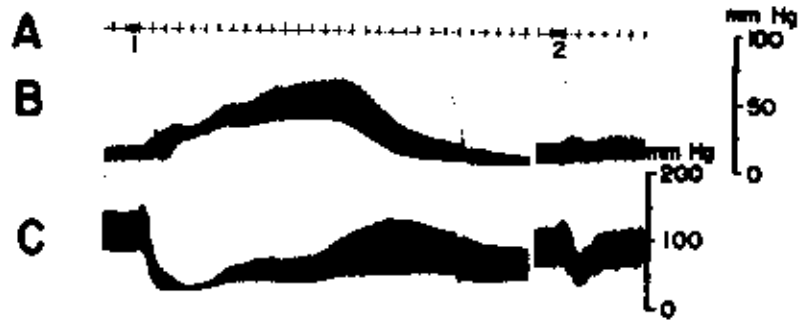

Fig. 3. *Effect of cottonseed oil emulsion on pressure in the pulmonary and carotid arteries.* Cat 3.3 kg, under chloralose anaesthesia with open thorax. A indicates 10-second intervals and injection times; B, the pulmonary artery; C, the carotid artery pressure. At 1 and 2 0.5 ml/kg of a 10% cottonseed 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

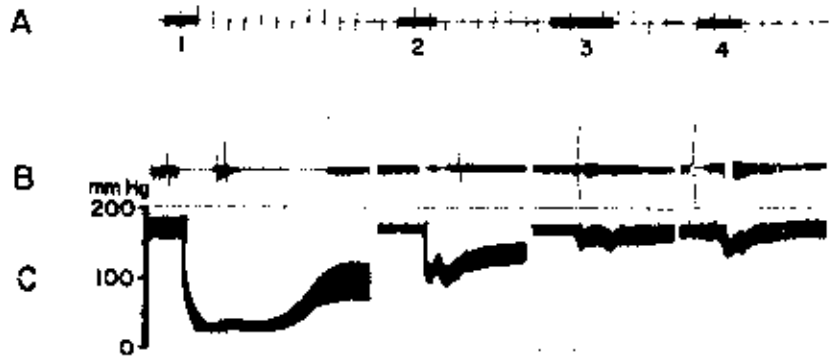

Fig. 4. *Effect of phosphatides on blood pressure and respiration.* Cat, 3.8 kg, under [illegible] anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, the [illegible] pressure in mm Hg. At 1, 2, 3 and 4 4 ml/kg of 2.4% solution of soya bean phosphatides and 0.2% Pluronic F 68 were injected. The phosphatides have a hypotensive action characterized by tachyphylaxis, though a slight effect persists.

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

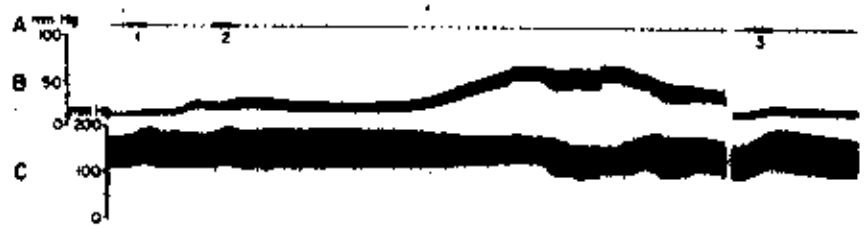

Fig. 5. *Influence of triolein emulsion on blood pressure and pulmonary arterial pressure after vagotomy.* Cat, 4.0 kg, under Dial E anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times: B, the pressure in the pulmonary artery; C, the arterial blood pressure in mm 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

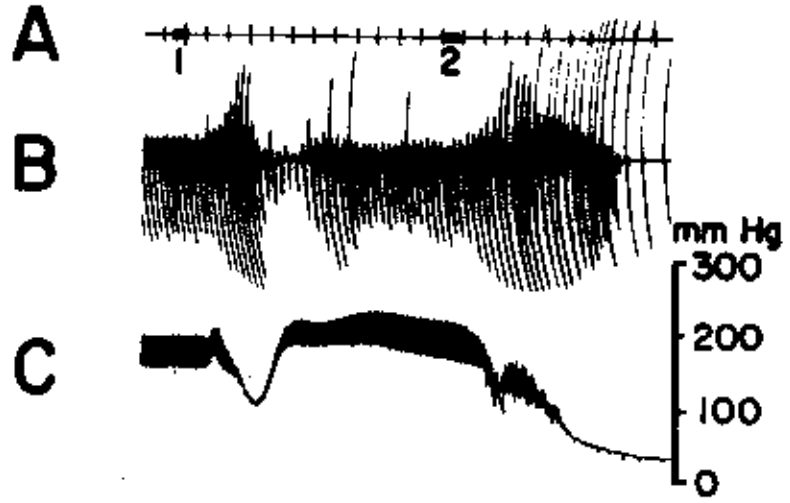

Fig. 6. *Effect of oleic acid emulsion on blood pressure and respiration.* Cat, 4.0 kg, under chloralose anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. One ml/kg of 10% oleic acid emulsion in cottonseed oil (10%) with 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 phospholipides (1.2%) and pluronic F 68 (0.2%) as emulsifiers injected at a dose of 0.2 ml/kg.

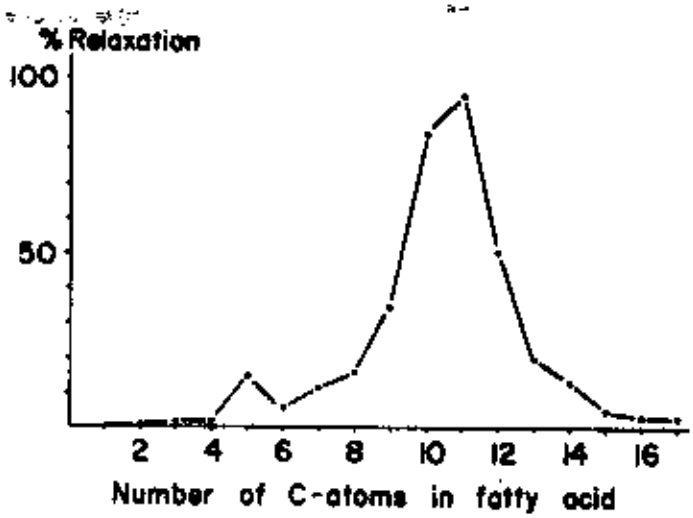

Fig. 8. *Relaxing action of fatty acids on guinea-pig small intestine contracted by histamine.* The fatty acids were administered in the form of emulsion. Abscissa indicates the number of carbon atoms in the fatty acid molecule. Ordinate shows percentage relaxation of guinea-pig intestine contracted 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 12 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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Determination of Digestibility and Metabolizable Energy in Pure Fats and Discussion of Analytical Methods Employed in Experiments with growing Chickens

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During recent years the question regarding the usability of fats as addition to feed mixtures has been of particular interest. There are three reasons for this: (1) The high energy content of the fats makes it possible to produce concentrated feed mixtures. (2) Fats which contain essential fatty acids, have a growth-promotive effect. (3) In the production of certain animal products it is possible to affect the composition of fatty acids in the product.

As far as poultry is concerned, addition of fat to the feed is used for all three reasons mentioned. For a complete utilization of the growth intensity of the broilers, it is necessary that the feed has a high energy content, on account of the high energy metabolism of the animals, compared to their weight. A supplement of unsaturated fat may come into consideration, when feed mixtures are used where the content of essential fatty acids is too low. Bælum & Petersen (1966) find a growth increase by addition of soya bean oil to a barley feed mixture, whereas no growth increase is found by addition of soya bean oil to a maize feed mixture. This is due to the fact that maize has a higher fat content than barley. Since poultry products have a high content of polyunsaturated fatty acids, they are well suited in the human nutrition for [illegible] the requirement for essential fatty acids. [illegible] been demonstrated that the content of polyunsaturated fatty acids in poultry products is easily increased by an addition to the [illegible] the fatty acids in question. Vestergaard Thomsen (1965) finds that egg fat is enriched with polyunsaturated fatty acids by feeding with soya bean oil and linseed oil.

During the last year a series of experiments with growing chickens has been conducted at this Department, partly in order to determine the content of metabolizable energy and the digestibility of pure fat, and partly to test the experimental procedure, with a view to forthcoming respiration experiments with growing chickens. In the experiment which will be discussed in the present paper, metabolizable energy as well as digestibility of lard, beef tallow, soya bean oil and linseed oil were determined. The tallow was obtained as cut tallow; it was thereafter ground and clarified, so that the product used was pure. The other three fats were obtained as pure fats.

Experimental plan

50-day-old chickens (White Plymouth Rock cocks) were obtained. For running the experiments 16 of the 50 were taken at random, after about 1 week they were placed individually in the experimental cages, and thus they were adjusted before the beginning of the experiment. The experiment started, when the chickens were 19-[illegible] to the beginning of the pre-experiment [illegible] the 16 chickens were distributed in four [illegible] group consisting of four [illegible]

[1] Norges fanthrakt[illegible]

Table [illegible] *[illegible] groups of the [illegible] at the beginning of the experiment*

[illegible]	1 Soya bean oil	2 Tallow	3 Lard	4 Linseed oil
[illegible]	267	281	255	309
	265	259	276	264
	262	251	292	276
	282	290	256	277
[illegible]	274	270	270	282
[illegible]	12	18	18	19

weights for the four groups were obtained. The weights of the four groups at the beginning of the experiment are given in Table 1.

Feeding plan

The experiment was carried through in five periods, and during the first and the fifth period a basal mixture was given. In the second, third and fourth period the basal mixture with added 6, 12 and 18% fat, respectively, was given. Thus a basal feed mixture and twelve feed mixtures with fat were prepared. A gradual feeding was used, the feed quantity being increased from day to day. This experimental method is described by Thorbek (1965). As the feed strength was further increased from period to period, the principle of gradual feeding was not followed [illegible] the feeding [illegible] strength then [illegible] pre-requisite [illegible] the full ration given in each feeding. Feed was given twice daily, at 8 a.m. and 4 p.m., and water was available *ad libitum*.

The feeding plan is represented graphically in Figure 1.

Composition of the feed

Large quantities of fat were added to the feed, which thereby became very rich in energy. For this reason, the basal feed mixture had to be enriched with protein. As protein sources were used soya bean meal and herring meal, to make sure that the protein had a high biological value. The composition of the basal feed is given in Table 2.

By feeding with large quantities of fat a vitamin-E deficiency may occur, and an extra supply of vitamin E was added. Similarly, the addition of antioxidant was meant to prevent oxidation during storage.

Collection of faeces

Every morning before the feeding the faeces was collected quantitatively in plastic boxes with covers, and stored in a freezer immediately after the collection. Nevertheless, a faint gas production could be observed in some of the samples: the loss must, however, be considered as negligible.

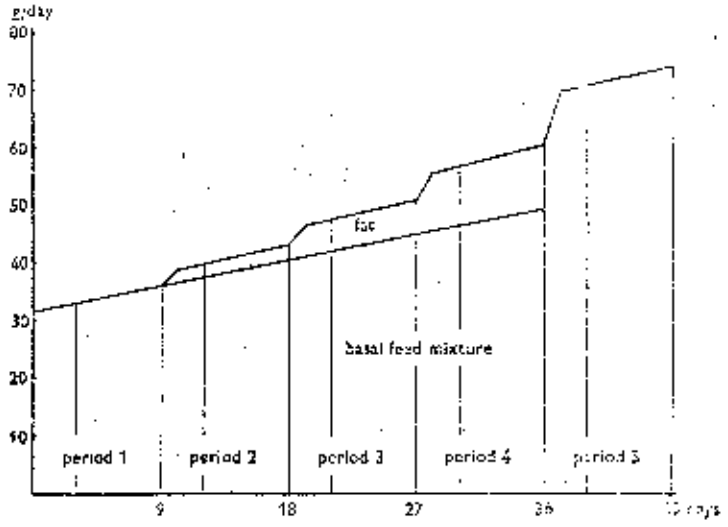

Fig. 1. Feeding plan.

[illegible] before taking out [illegible] the [illegible] in an [illegible] for determination [illegible] nitrogen and [illegible] of the faeces were [illegible] for determination of fat and energy. [illegible] analyses mentioned were made in accordance with the analytical precepts of the laboratory (Weidner & Jakobsen, [illegible]). As this experiment primarily serves to determine [illegible] energy in fat and the digestibility of fat, the energy determination and the fat analyses will be mentioned more detailed and discussed on the basis of the results of the experiments.

Energy determination

The determination of the energy in feed and faeces was made on an adiabatic calorimeter (Gallenkamp). The principal difference between an adiabatic calorimeter and the older calorimeters of the Department is that the water jacket surrounding the calorimeter [illegible], by means of thermostatic [illegible] with the use of temperature in the calorimeter [illegible] so that no heat exchange with the environment takes place. Similarly, the reading work is facilitated, as the temperature is [illegible] twice, as compared with 8–10 times for the older calorimeters. In the determinations of energy in the pure fat, the fat was weighed in the crucible, and on the ignition wire a small cotton thread (0.004–0.005 g) was placed, which served as preservation of the fat. [illegible] this arrangement [illegible] difficult to ignite the pure fat samples.

[illegible] Composition [illegible] percent

Barley	[illegible]
Maize	[illegible]
Soya bean meal	[illegible]
Herring meal	[illegible]
Vitamin mixture*	[illegible]
Dicalcium phosphate	[illegible]

Crude protein: 24.4%. Crude fat: 3.0%
Gross energy: 4.431 kcal/g dry matter

* Composition of the vitamin mixture, 1 g [illegible] vitamin A, 1000 I.U.; vitamin D_3, 400 I.U.; vitamin [illegible] 200 µg; vitamin B_1, 640 µg; nicotinamide, [illegible] µg; [illegible] acid, [illegible] µg; α-tocopherol-acetate, [illegible] µg; 1-2-dihydro-6-ethoxytrimethylquinoline, [illegible]

Analyses were made on all feed mixtures. It appeared that the results from the analysis were somewhat lower than the calculations made on the basis of basal feed and the pure fat, respectively. The reason for this may be ascribed to the fact that the pelleting of the very fatty samples prior to burning, may squeeze out fat. Therefore, the energy content of the feed was calculated on the basis of energy determinations in the basal feed and the pure fat separately. The results of the analyses of the feed mixtures and the values calculated on the basis of analyses on basal feed mixture and fat, separately, are given in Table 3.

Energy content of the fat

Soya bean oil:	9.430 kcal/g
Tallow:	9.484 kcal/g
Lard:	9.424 kcal/g
Linseed oil:	9.430 kcal/g

Table 3. *Energy content of the feed mixtures (in kcal)*

	Soya bean oil		Tallow		Lard		Linseed oil	
	Total mixture analysed	Basal mixture and fat analysed	Total mixture analysed	Basal mixture and fat analysed	Total mixture analysed	Basal mixture and fat analysed	Total mixture analysed	Basal mixture and fat analysed
	Feed							
Basal mixture + 6% fat	4.691	[illegible]	4.726	[illegible]	[illegible]	[illegible]	4.726	[illegible]
Basal mixture + 12% fat	5.028	5.138	[illegible]	[illegible]	[illegible]	[illegible]	5.605	5.147
Basal mixture + 18% fat	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		

[illegible] coefficients for crude fat

	Chicken no.	Basal mixture Period 1	Period 5	[illegible] mixture [illegible] 6% fat Period 2	Basal mixture + 12% fat Period 3	Basal mixture + 18% fat Period 4
[illegible]	1	78.0	75.8	90.8	95.8	96.5
	2	80.1	78.1	92.4	95.4	95.7
	3	79.5	77.5	91.8	94.0	95.2
	4	80.2	76.3	92.0	95.9	96.2
	5	79.6	79.6	81.8	—[a]	91.7
	6	75.4	77.1	86.1	83.8	87.7
	7	76.3	77.4	86.9	88.0	91.6
	8	73.8	76.9	85.9	87.2	83.8
[illegible]	9	77.1	78.4	90.3	92.6	92.7
	10	76.4	77.3	88.9	93.3	89.5
	11	78.4	76.7	85.1	94.6	92.5
	12	74.3	74.3	88.4	93.5	90.2
[illegible]	13	79.2	76.7	89.3	94.2	96.1
	14	75.4	76.6	91.7	92.3	95.4
	15	79.9	76.8	93.3	95.4	96.0
	16	79.6	77.5	92.2	94.4	96.1
	$\bar{x}$	77.7	77.1	89.2	92.3	92.9
[illegible] deviation		2.2	1.2	1.8	1.1	2.1
[illegible] deviation p.c.		2.8	1.6	2.0	1.4	2.3

[a] Fat analysis lost.

[illegible] figures stated in Table 3 show the condition [illegible] differed, and it seems further that the difference [illegible] test with liquid fats. As for the faeces, both [illegible] determinations and fat analyses were [illegible] on the freeze-dried samples.

Fat analyses

The accuracy for determining digestibility of [illegible] depends as well on the storage of the analytical [illegible] and their preparation prior to the analys[illegible] on the analytical method itself. Both during the storing of the samples and during the drying the fat fraction may undergo considerable changes. By a heat-drying of the wet material [illegible] to the analysis an oxidation of the fat may [illegible] place. Such an oxidation will especially occur at the double linkages in the carbon chain [illegible] the unsaturated fatty acids, and in the current [illegible] oxidation will [illegible] (1933) [illegible] on

where the analyses are made on freeze-dried samples, the standard deviation of the digestibility is lower. It lies between 1.5%–3% as well for the basal feed mixture with the low fat content as for the fatty mixtures. The digestibility coefficients for crude fat are given in Table 4.

From Table 4 it will be seen that the digestibility coefficients for the basal feed are from 77.1–77.7, and that the digestibility in the periods 2, 3 and 4 is considerably higher.

There is no reason to assume that the fat in the basal feed has a lower true digestibility than the fat added, but since some endogenous fat is excreted, and part of the bile [illegible] are dissolved by the extraction, the apparent digestibility will be lower when the fat quantities in the feed are small.

[illegible] 1 the [illegible] 3.5 [illegible] feed. [illegible] (1933) [illegible] feed as [illegible]

Table 5. [illegible] and digested quantities of pure fat

		Period 1	Period 2	Period 3	Period 4
Consumed fat, g/day		[illegible]	2.61	5.97	10.59
		Digested fat, g/day			
Soya bean oil	1		2.44	5.58	10.43
	2		2.47	5.62	10.31
	3		2.45	5.72	10.26
	4		2.46	5.86	10.36
Tallow	5		2.08	—	9.52
	6		2.27	5.09	9.48
	7		2.29	5.39	9.95
	8		2.24	5.15	9.02
Lard	9		2.41	5.72	10.02
	10		2.37	5.78	9.65
	11		2.22	5.87	10.00
	12		2.38	5.83	9.77
Linseed oil	13		2.36	5.83	10.37
	14		2.47	5.72	10.31
	15		2.50	5.91	10.36
	16		2.46	5.83	10.37

in order to prevent a possible oxidation during the storing of the samples.

A reason for the change in the level of digestibility coefficients and a change in the standard deviation may be ascribed to the analytical method itself. It may here be a question of using different extraction methods, where the different fat fractions have a different solubility. Vestergaard Thomsen (1965) has investigated the difference in solubility of fat from chicken faeces by ether extraction and by chloroform extraction, and finds that more crude fat is dissolved by chloroform extraction, and finds consequently lower digestibility coefficients. Part of this difference may be due to the greater solubility of the bile pigments in chloroform.

The fat analyses are carried out in accordance with the precepts of the Department, with the modification that the samples are dried to constant weight under vacuum and nitrogen flow for 22 hours prior to the extraction. This analytical technique is described by Weidner (1967). Just Nielsen (1967) finds a difference in the digestibility of crude fat in pigs, where the fat analyses on the [illegible] parts made in accordance with the precepts of the Department, and partly after drying for 22 hours under vacuum and nitrogen flow prior to the extraction. In the analyses made according to this last-mentioned method, [illegible] to 72.7, because a smaller quantity of crude fat was found in the faeces, and likewise the standard deviation of the digestibility coefficients decreased. These analyses were made on heat-dried samples. The conclusion of the above described conditions is that freeze-drying of the samples compared with heat-drying reduces the standard deviation of the digestibility coefficients and that the digestibility coefficients are depending on the analytical method used.

Another factor which affects the digestibility of the fat is the calcium content of the feed. Pettersson (1964) finds, in experiments with pigs that the digestibility of the fat decreases with an increasing calcium content in the feed, as a precipitation of the calcium salts of the fatty acids takes place in the digestive tract, and finds that such a saponification occurs too, when the feed has a normal content of calcium. This condition particularly applies to the long-chained saturated acids: myristic acid, palmitic acid and stearic acid, whereas the digestibility of oleic acid, linolic acid and linolenic acid is only affected to a smaller degree. By the usual fat analysis with ether extraction the precipitated calcium salts are withdrawn, due to their insolubility in ether, and the fat content in the faeces is determined too low. Hereby, a higher digestibility coefficient will be determined, despite of the lower digestibility of the fat. In the experiments carried out by Pettersson, the fat extraction by the analysis was made after a preceding hydrolysis with 7.5 *n* hydrochloric acid.

Digestibility of the pure fats

On the basis of the digested quantities of crude fat in the basal feed and the digested quantities in the total feed, the digested crude fat from the four different kinds of fats has been calculated for each chicken separately in the three periods. The consumed and the digested quantities of the four kinds of fats are given in Table 5.

On the basis of the figures given in Table 5 the digestibility coefficients are calculated for the individual fats according to the regression method, and are given in Table 6.

Table 6. *Digestibility coefficients for the pure fat*

	[illegible]	[illegible]
Soya bean oil	97.7	0.5
Beef tallow	91.3	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

[illegible] per g consumed dry matter (Kcal)

	Chicken no.	Period 1 Basal mixture	Period 2 Basal mixture + 6% fat	Period 3 Basal mixture 12% fat	Period 4 Basal mixture 18% fat	Period 5 Basal mixture
		Kcal				
Soya bean oil	1	3.348	3.769	4.142	4.552	3.324
	2	3.267	3.747	4.150	4.525	3.358
	3	3.289	3.715	4.099	4.501	3.298
	4	3.343	3.766	4.159	4.570	3.330
Tallow	5	[illegible]	3.692	3.951	4.182	3.311
	6	3.303	3.606	3.961	4.092	3.362
	7	3.338	3.713	3.907	4.1[illegible]5	3.347
	8	[illegible]	3.650	3.870	4.015	3.359
Lard	9	3.304	3.755	4.031	4.373	3.337
	10	3.298	3.720	4.045	4.237	3.337
	11	3.328	3.731	4.072	4.357	3.317
	12	3.289	3.743	4.036	4.322	3.337
Linseed oil	13	3.359	3.694	4.171	4.545	3.355
	14	3.271	3.646	4.103	4.472	3.292
	15	3.302	3.750	4.194	4.597	3.313
	16	3.273	3.554	4.147	4.556	3.317
$\bar{x}$		3.306	3.703	4.058	4.371	3.338
Standard deviation		0.029	0.050	0.027	0.053	0.025
Standard deviation p.c.		0.9	1.4	0.7	1.2	0.7

It will be seen that the digestibility coefficients are highest for the two liquid fats, soya bean oil and linseed oil. These two have the highest content of unsaturated fatty acids. The other two fat sources, tallow and lard, have a lower digestibility due to a high content of palmitic and stearic acid.

The standard deviation is highest for tallow [illegible] due to the [illegible] in obtaining a [illegible] homogeneous mixtures of the basal [illegible] these two fats. [illegible]

The content of metabolizable energy

In the first and the fifth period of the experiment [illegible]

differences, it was sufficient to correct to a fixed nitrogen deposition. For a deposition of 45%, only small corrections were necessary. The metabolizable energy per g dry matter, corrected to 45% nitrogen deposition, is stated in Table 7.

[illegible] the periods 2, [illegible] and [illegible] is the lower standard deviation.

From the low standard deviations of the metabolizable energy it will be seen that the energy determinations are rather safe analyses. In digestibility experiments with pigs, Madsen [illegible] the determination of the digestible energy [illegible] deviation. Similar results [illegible] by Gad [illegible] Thomsen (1967) in [illegible] experiments with hens.

[illegible] from the [illegible] the [illegible] of the [illegible]

Table 8. *Metabolizable energy in the pure fat (in kcal)*

	Chicken no.	Period 2	Period 3	Period 4
Consumed fat, g/day ...		2.51	8.97	10.39
		Metabolizable energy, kcal/day		
Soya bean oil	1	24.22	55.[illegible]	97.75
	2	24.43	53.15	97.53
	3	23.73	55.[illegible]	96.91
	4	24.17	[illegible]	98.67
Tallow	5	17.59	[illegible]	82.62
	6	19.19	[illegible]	76.42
	7	21.81	41.19	79.86
	8	19.70	43.49	72.17
Lard	9	24.04	51.04	90.01
	10	23.04	51.51	84.19
	11	24.08	53.75	89.24
	12	23.82	51.52	87.30
Linseed oil	13	22.74	55.95	97.29
	14	21.79	54.13	94.42
	15	26.46	57.81	97.30
	16	23.14	54.23	98.85

the metabolizable energy per g fat is calculated by means of a regression analysis. The results are given in Table 9.

The results show that the metabolizable energy for soya bean oil and linseed oil is very high, which corresponds with the high digestibility coefficients for these fats. The metabolizable energy is lower for lard, and lowest for tallow.

In Table 10 the direct determined digestibility coefficients are compared with the digestibility coefficients calculated after the metabolizable energy.

By a comparison between the digestibility coefficients and the metabolizable energy it appears that, as far as lard and tallow are concerned, the digestibility coefficients are higher, whereas there is no difference as regards linseed oil and soya bean oil. This difference may be due to the fact that, as far as fatty acids are concerned, a precipitation of saturated fatty acids of the fatty acids in the stable and urine takes place, and that the fat content of the faeces has thus been determined [illegible] the analytical method used.

Table 9. *Metabolizable energy per g pure fat*

	kcal/g	s_b	s
Soya bean oil	9.10	0.05	0.67
Beef tallow	7.05	0.24	0.50
Lard	7.52	0.15	1.23
Linseed oil	9.07	0.18	2.11

Table 10. *Digestibility coefficients direct determined versus digestibility coefficients calculated after the metabolizable energy*

	Soya bean oil	Beef tallow	Lard	Linseed oil
Determined	[illegible]	91.3	92.7	97.8
Calculated	[illegible]	74.3	81.0	96.1

Summary

Experiments have been carried out for determination of the metabolizable energy as well as for the digestibility of four pure fats: Soya bean oil, beef tallow, lard and linseed oil. The analysis of the faeces are made on oven-dried samples. By a comparison with coefficients calculated by other methods, freeze-drying versus oven drying of samples of faeces causes no [illegible] change in the digestibility coefficients as a result. [illegible] standard deviation. The metabolizable energy is higher, as far as the two liquid fats are concerned. For the other two fats, there is a difference between the metabolizable energy and the digestibility coefficients. The digestibility coefficients for tallow and lard are found to be too low in relation to the metabolizable energy. This condition may be due to a precipitation of saturated fatty acids in the urine, which are [illegible] when the values are calculated from the determined analytical method.

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3 *Physiology*

STEARIC ACID AS A POISON G. E. Price* and R. H. Heutner
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The toxicity of the higher fatty acids is believed to be low, but we found that they have a demonstrable toxicity when used in high doses. The method of Dr. Philip Harris of D.P.I. Rochester, N.Y. was used. The experiments were done on young white rats housed in individual cages at a temperature of 21°C. One half of the total food was stearic acid. The remainder was casein, glucose, salt mixture, cellulose and vitamin mixture. Water was supplied ad lib. The male rats died after an average of 8.2 days, the female rats after 10.2 days. Shortly before death the rats showed cardiac irregularities, demonstrable on the E.K.G. In some cases there was spasm and paralysis of the extremities. In other experiments the rats were given 3/10 the dose of stearic acid, in these cases death was very considerably delayed. The toxicity of stearic acid was markedly diminished by addition of glyceride oil, such as corn oil, even if as little as 5% was added.

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NUTRITIONAL EFFECTS OF INCLUDING LOW LEVELS OF TALLOW AND PALM-KERNEL OIL IN ALL CONCENTRATE AND CONCENTRATE/HAY DIETS FOR YOUNG CATTLE

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An experiment was carried out to study the effect on digestibility of dietary constituents and retention of nitrogen of including tallow and palm-kernel oil in a dry concentrate mixture for calves. The mixtures compared were a control and two others containing 5 per cent of tallow and palm-kernel oil respectively. During part A of the experiment the comparison was made without supplementary feeding, and during part B hay was also provided so that the proportion of concentrates to hay was two to one.

In both parts of the experiment the inclusion of fat did not reduce the digestibility of organic matter or that of any individual constituent of the organic matter. The digestibility of total dietary lipid was increased significantly, and the calculated digestibilities of the tallow and palm-kernel oil were 87.6 and 97.1 per cent during part A and 90.5 and 96.2 per cent during part B of the experiment. The inclusions of fat were associated with higher retentions of nitrogen, the mean retentions being 33.7, 37.0 and 39.9 per cent of intake during part A, and 33.5, 37.8 and 40.4 per cent of intake during part B, for the control, tallow and palm-kernel oil treatments respectively.

INTRODUCTION

The present trend towards more intensive systems of rearing cattle, particularly for beef production, has led to a greater recognition of the importance of dietary energy in determining rate of growth and efficiency of animal production. This need for energy is particularly evident with the young calf because of a change in feeding practice from extended feeding of whole milk with its very high energy content to the use of milk replacers based largely on low-fat milk by-products, and the more recent development of early weaning onto dry concentrates and hay. However, studies of the nutritive value of different fats in milk replacers for calves (Raven and Robinson 1958, 1959, 1960, 1964a) and of the effect of different methods of incorporation of the fat in the replacer on its subsequent nutritive value (Raven and Robinson 1964a), have shown that fats such as tallow and certain vegetable oils can be used to substantially increase the energy value of milk replacers. The most recent trend in feeding practice has therefore been an increasing use of fat in milk replacers, and a consequent increase in their energy value.

This knowledge that the young calf being fed either whole milk or a high-fat milk replacer is accustomed to a high-fat diet raises the possibility that when given a diet of dry concentrates and roughage, it might continue to receive a supplementary level of fat in its diet. This possibility of providing additional fat is particularly pertinent when one considers that increasing use of barley, which contains a relatively low level of fat compared with other cereals, and of solvent extracted protein supplements are both tending to reduce the level of fat in concentrate mixtures for cattle. As fat contains approximately 2¼ times as much gross energy per unit weight as does carbohydrate its potential value as a means of increasing the energy value of concentrate mixtures is obvious, but it could also serve a useful purpose in reducing the dustiness of feeds and consequent loss of dusty material.

The effect of including 2.5, 5.0 and 10.0 per cent of stabilised tallow in a calf starter was studied by Johnson, Jr., Dolge, Rousseau, Jr., Teichman, Eaton, Beall and Moore (1956). In this study the calves were fed limited whole milk to 35 days of age, a maximum of 4 lb per day of starter, and alfalfa pellets *ad libitum*. Performance of the animals to 92 days of age showed that those fed starters containing tallow had a 5 to 6 per cent greater rate of growth and required less food dry matter per lb of liveweight gain. In 1959, Miller, Carmon and Dalton reported finding that the addition of 10 per cent brown grease, 10 per cent hydrogenated cottonseed oil, or 5 per cent of each to calf starters markedly reduced food consumption and liveweight gains. However, in a subsequent study Miller (1962) reported finding that the addition of 10 per cent butter, tallow, lard or hydrogenated cottonseed oil to starters did not significantly affect liveweight gains over an 8 week growth trial which commenced when the calves

were 6 days old. In this study the mean consumption of starter and rate of gain were each highest on the starter containing butter and lowest on the one containing hydrogenated cottonseed oil, with the one containing tallow giving results similar to those on the control starter. The relative palatibilities of the starters were further assessed in subsequent trials where calves were offered a choice of starters, and it was found that calves preferred the control starter to those containing the added fats.

It is now well established, as indicated in a recent review of the digestion and assimilation of lipids (Garton 1965), that rumen microorganisms can effect hydrogenation of unsaturated fatty acids to produce saturated fatty acids. As the amount of saturated fatty acids present in a fat and also chain length of the constituent fatty acids are both considered to affect the digestibility of fats by monogastric animals, a comparison is made in the present experiment of two fats of contrasting fatty acid composition, namely tallow and palm-kernel oil. The latter fat was used because it contains a much higher proportion of short-chain fatty acids, notably lauric and myristic acids, than does tallow (Hilditch and Williams 1964). Furthermore, in view of the evidence that a 10 per cent level of inclusion of fat can have an adverse effect on palatability of the diet the comparison in the present study was made at a level of inclusion of 5 per cent.

EXPERIMENTAL

DIETS

The concentrate mixtures used were of high protein content and were made up as shown in Table 1. The expanded maize, which was used as a carrier for the tallow and the palm-kernel oil, was prepared commercially by subjecting maize grain to short-term pressure cooking followed by drying and coarse grinding. This gave a low-density product with a high absorptive capacity for fat. In order to prevent any reduction in crude protein content through the inclusion of fat in place of rolled barley, the two mixtures containing added fat also contained slightly higher percentages of soya bean meal and decorticated earthnut meal.

The experiment was divided into two parts, A and B. Part A consisted of digestibility and nitrogen balance trials in which the three concentrate mixtures were compared without any supplementary feeding of roughage. Immediately following completion of part A the calves were continued on the same intake of concentrates and also given hay so that the proportion of concentrates to hay was two to one. Further digestibility and nitrogen balance trials were then carried out to study the effect of also feeding hay in conjunction with the concentrate mixtures. The concentrate mixtures were fed twice daily, at 9 a.m. and 5 p.m., and hay when fed was provided once daily at 10 a.m.

TABLE 1

Percentage composition of the concentrate mixtures

Ingredient	*Treatment 1 (control)*	*Treatment 2 (tallow)*	*Treatment 3 (palm-kernel oil)*
Rolled barley	65	58	58
Expanded maize	10	—	—
High-fat expanded maize *	—	15	15
Soya bean meal (extracted)	10	11	11
Decorticated earthnut meal	10	11	11
White fish meal	5	5	5
Mineral, vitamin, and chlortetracycline supplements †	+	+	+

* The high-fat expanded maize consisted of 67 per cent expanded maize, 31 per cent stabilized fat and 2 per cent lecithin supplement, the fat used being tallow for treatment 2 and palm-kernel oil for treatment 3.

† Chlortetracycline was included in each mixture at a level of 40 mg per lb of meal. Dry vitamin supplements were also added to supply 2,400 i.u. of vitamin A, 600 i.u. of vitamin D_2 and 5 mg of vitamin E per lb of meal. At the time of feeding 5 ml of a solution of B-vitamins made up as previously described (Raven and Robinson 1959) and 5 ml of a solution of trace minerals made up according to the formula of Blaxter and Wood (1952) were added to each feed.

PLAN OF EXPERIMENT

For the experiment six Friesian bull calves were weaned abruptly at four to five weeks of age and housed in conventional metabolism crates. They were then allocated to two 3 × 3 Latin squares and fed the appropriate concentrate mixtures. Each experimental period consisted of a six-day preliminary feeding period followed by an eight-day balance period, the first balance period commencing when the calves were six weeks of age. The daily allocation of concentrate mixture was 1,000 grams given as two equal feeds. Immediately following the three periods of part A of the experiment the procedure was repeated, the only differences being that the calves were twelve weeks old at the commencement of this part and were given 500 grams of chopped hay per day in addition to the allowance of 1,000 grams of concentrate mixture. The total allocations of food were fully consumed in both parts of the experiment except for a few instances during part A when small amounts of residue were left and had to be taken into account in the calculation of results. In each part of the experiment a fresh supply of water was provided each day on an *ad libitum* basis. The results obtained in each part of

the experiment were analysed as two 3 × 3 Latin squares, the nature of the treatments and intervening periods between balance periods being such that carry-over effects could be regarded as negligible.

All the animals remained in good condition throughout the balance periods and in no instance was any scouring observed.

COLLECTION AND ANALYSIS OF SAMPLES

Daily collections of faeces and urine were made during the balance periods. A constant proportion of the weight of wet faeces from each animal was retained and stored in a plastic container in a refrigerator. Likewise, a definite fraction of each days output of urine was taken and bulked. Nitrogen determinations were made on the composite wet samples of faeces and bulked samples of urine immediately after the end of each balance period, and the dry matter contents of the faeces ascertained. All other analysis of faeces was carried out on composite samples of dried faeces.

Before chemical analysis the samples of concentrate mixtures, hay and dried faeces were milled to pass through a $\frac{1}{24}$ inch sieve. The dry matter, crude protein (N × 6.25), crude fibre and ash constituents were determined by the usual methods of proximate analysis. However, as the normal Soxhlet procedure for determining ether extract does not achieve complete extraction of faecal lipids, the determination of total lipid in both food and faeces was made by a method similar to one used for the determination of fat in dried milk (British Standards Institution, 1951). This method involves treatment with hydrochloric acid before extraction of the lipids and thus enables any fatty acids present as soaps to be converted to free fatty acids and therefore extracted. The percentage of nitrogen-free extractives in the dry matter was obtained by summating the percentages of crude protein, crude fibre, total lipid and ash in the dry matter and subtracting from 100.

RESULTS

CHEMICAL COMPOSITION OF THE CONCENTRATE MIXTURES AND HAY

It can be seen from Table 2 that the crude protein contents of the concentrate mixtures were very similar. The inclusion of tallow and palm-kernel oil in the concentrate mixtures of treatments 2 and 3 respectively gave very similar levels of total lipid in these two mixtures. In comparing the chemical composition of the three concentrate mixtures it is evident that the increased level of fat in the high-fat mixtures was achieved by substitution for nitrogen-free extractives, the percentages of all other constituents being very similar in the three mixtures. The analysis of the hay indicates that it was of good quality, having a high content of crude protein and a reasonably low content of crude fibre.

TABLE 2

Mean chemical composition of the concentrate mixtures and hay (as per cent of the dry matter)

	Concentrate mixtures			*Hay*
	Treatment 1 (control)	*Treatment 2 (tallow)*	*Treatment 3 (palm-kernel oil)*	*All treatments where fed*
Organic matter	95.5	95.4	95.4	92.2
Crude protein	23.8	24.1	25.1	13.0
Total lipid	3.61	8.49	8.43	2.38
Crude fibre	4.02	4.03	3.85	33.5
N-free extractives	64.1	58.8	58.0	43.4
Ash	4.48	4.63	4.65	7.77

DIGESTIBILITY COEFFICIENTS

The results (Table 3) show that the digestibility of the organic matter was slightly higher for treatments 2A and 3A than for treatment 1A, but the differences were not significant. The higher digestibilities on the high-fat treatments were evidently the result of higher digestibilities on these treatments of both crude protein and total lipid, the nitrogen-free extractive fraction showing little difference between treatments. The mean level of crude fibre in the dry matter of the diets without supplementary roughage was only 4.0 per cent and considerable variation occurred in its digestibility within each treatment. However the mean results indicate there was little difference between treatments.

The feeding of hay along with the concentrate mixtures (treatments 1B, 2B and 3B) led to a substantial reduction in the digestibility of organic matter, but results for the three treatments were very similar. Although the digestibilities of crude protein and total lipid were again higher on the high-fat treatments, only in the latter case did the increase attain significance. Results for the nitrogen-free extractives fraction were again very similar on all three treatments. The higher crude fibre contents in the concentrate/hay diets (mean value 13.9 per cent in the overall dry matter) than in the all concentrate diets were associated with substantially higher digestibilities for this fraction

TABLE 3

Mean percentage apparent digestibility of the dietary constituents

Constituent	*Treatment 1A (control)*	*Treatment 2A (tallow)*	*Treatment 3A (palm-kernel oil)*	*Standard error of a mean*	*Significance of treatment differences*
Organic matter	82.0	83.1	83.5	0.68	N.S.
Crude protein	73.7	77.7	78.5	1.35	N.S.
Total lipid	51.1	72.8	80.2	2.14	*** 2A and 3A > 1A * 3A > 2A
True digestibility of added fat	—	87.6	97.1	—	—
Crude fibre	33.6	37.1	32.0	3.70	N.S.
N-free extractives	89.0	89.2	89.1	0.38	N.S.

	Treatment 1B (control)	*Treatment 2B (tallow)*	*Treatment 3B (palm-kernel oil)*	*Standard error of a mean*	*Significance of treatment differences*
Organic matter	77.6	77.6	77.0	0.77	N.S.
Crude protein	74.5	76.3	77.2	0.88	N.S.
Total lipid	64.7	78.8	83.0	0.77	*** 2B and 3B > 1B ** 3B > 2B
True digestibility of added fat	—	90.5	96.2	—	—
Crude fibre	59.5	59.0	54.7	1.56	N.S.
N-free extractives	82.8	82.0	81.6	0.54	N.S.

in part B of the experiment. The lower digestibility of the organic matter on treatments 1B, 2B and 3B than on treatments 1A, 2A and 3A is evidently the result of the higher level of crude fibre with its lower digestibility than those of other constituents, and a reduction in the digestibility of the nitrogen-free-extractives fraction associated with the inclusion of hay.

NITROGEN RETENTION

It is evident from the mean results for retention of nitrogen (Table 4) that the inclusion of tallow and of palm-kernel oil were each associated with higher retentions of nitrogen than were obtained on corresponding control treatments. This improvement in nitrogen retention was significant in part B of the experiment when hay was being provided. Although improvements were also evident in the absence of hay they were not as great and the results were more variable. The differences between the mean retentions of nitrogen in part A of the experiment did not therefore attain significance.

TABLE 4

Mean retention of nitrogen

	Treatment 1A (control)	*Treatment 2A (tallow)*	*Treatment 3A (palm-kernel oil)*	*Standard error of a mean*	*Significance of treatment differences*
Intake (g per day)	32.3	33.0	33.6	—	—
Retention (g per day)	10.9	12.2	13.5	0.99	N.S.
Retention (per cent of intake)	33.7	37.0	39.9	2.35	N.S.
Biological value of crude protein (per cent)	67.1	68.2	69.3	2.15	N.S.

	Treatment 1B (control)	*Treatment 2B (tallow)*	*Treatment 3B (palm-kernel oil)*	*Standard error of a mean*	*Significance of treatment differences*
Intake (g per day)	42.5	43.3	44.8	—	—
Retention (g per day)	14.4	16.3	18.1	0.50	** 3B > 1B * 2B > 1B * 3B > 2B
Retention (per cent of intake)	33.5	37.8	40.4	1.41	* 3B > 1B
Biological value of crude protein (per cent)	65.8	68.9	70.1	1.48	N.S.

The biological values of the crude protein provided on each treatment were calculated using recommended factors (Agricultural Research Council 1965) for estimating metabolic faecal nitrogen and endogenous urinary nitrogen. Although the values obtained on the control treatments were rather lower than 70 per cent, which has been suggested as a mean value for protein in diets given to cattle (Agricultural Research Council 1965), they do indicate a satisfactory level of efficiency of utilisation of absorbed nitrogen. The results obtained on the high-fat treatments were, however, slightly higher, suggesting that the inclusion of additional fat in the diet led to some improvement in utilisation of absorbed nitrogen.

DISCUSSION

In considering the digestibility of fat in dry concentrate mixtures for calves it is important to appreciate the physiological and biochemical changes which occur with a change in feeding from liquid milk or milk replacer to dry concentrates and possibly roughages. When the young calf is given a liquid milk or milk replacer diet a sucking mechanism operates and the milk by-passes the rumen and reticulum, via the oesophageal groove, and enters directly into the abomasum or true stomach. The food therefore avoids fermentation in the rumen and is enzymically digested in the abomasum and intestines. However, when the calf is given dry concentrates or roughages the food enters the rumen and is subjected to fermentation before absorption.

The effect of providing food in dry form as compared with liquid form has been previously studied and discussed (Raven and Robinson 1964b). In addition the changes in dietary fat which take place during passage through the rumen have been recently reviewed (Garton 1965); two of the more important changes which occur are that rumen microorganisms effect hydrolysis of dietary fat thereby releasing fatty acids, and they also effect considerable hydrogenation of unsaturated fatty acids to produce saturated acids. Any glycerol released from lipids in the rumen is rapidly fermented to volatile fatty acids of which propionic is the main product. However, it seems that there is no appreciable degradation of long-chain fatty acids to steam-volatile breakdown products. This conclusion is borne out by the fact that change from liquid feeding to dry feeding of a high-fat diet does not cause any reduction in the level of esterified fatty acids in plasma whereas change from a high-fat to a low-fat diet leads to a rapid decline in the level of esterified fatty acids Raven and Robinson 1964b). It is also supported by the findings of Esplin, Hale, Hubbert, Jr. and Taylor (1963) who reported that addition of 4 per cent of tallow to a fattening ration for 830 lb Hereford steers did not significantly affect the molar ratios of steam-volatile fatty acids in the rumen fluid. In his review Garton points out that although short-chain water-soluble fatty acids are readily absorbed through the rumen wall, very little absorption of long-chain fatty acids apparently takes place. The relative amounts of lipids and their fatty acid composition do not change markedly during passage through the omasum and abomasum and the end result is that of a more or less continuous passage of lipid material to the duodenum and jejunum. This lipid material contains a considerable proportion of free, long-chain fatty acids which are presumably taken into micellar solution by bile salts and thereby absorbed.

The true digestibilities of the tallow and palm-kernel oil were calculated by using the values for food and faecal lipids obtained on the control treatments to estimate the food and faecal lipids associated with the basal constituents of the high-fat diets. The results indicate that although both fats were well digested, the mean digestibility of the palm-kernel oil was appreciably higher than that of the tallow. Assuming effective lipolysis in the rumen, which would serve to eliminate differences in triglyceride structure, the higher digestibility of the palm-kernel oil must represent a higher absorption of its constituent fatty acids than those of tallow. It seems therefore that relatively short-chain fatty acids, such as lauric and myristic acids, are better absorbed than longer chain fatty acids. The true digestibilities of 87.6 and 90.5 per cent obtained for the tallow in parts A and B of the experiment are slightly lower than the value of 93.4 per cent reported by Esplin *et al* (1963) for a 4 per cent level of inclusion of tallow in a beef fattening ration. However, higher levels of fat in dry meal mixtures than the 5 per cent level of addition used in the present experiment have given much poorer digestibilities. Raven and Robinson (1964b) reported a digestibility of 67.4 per cent for the total lipid in a diet containing 26 per cent of palm oil, and 63.6 per cent for the total lipid of a meal mixture containing 10 per cent of added tallow. It seems that, in addition to an adverse effect on palatability of the diet, levels of added fat of 10 per cent and more in dry concentrate mixtures are associated with appreciably lower digestibility of the dietary lipid than is obtained with a 4 or 5 per cent level of inclusion of fat. It is of interest that with liquid diets containing high levels of fat, homogenisation of the fat gives a substantially higher digestibility for it than is obtained by blending with separated milk powder

followed by reconstitution in water, but with dry foods the inclusion of fat in the form of an homogenised spray dried milk powder as compared with blending does not result in a corresponding improvement in digestibility of the fat (Raven and Robinson 1964b). The lower digestibility of high levels of fat in dry concentrate mixtures was further clarified in the same studies by the finding of a substantial reduction in digestibility of the lipid material in high-fat diets when a change was made from liquid to dry feeding.

In addition to a consideration of the digestion and absorption of the added fat, consideration must also be given to its possible effects on the digestibility of other constituents. Johnson *et al* (1956) reported that the inclusion of tallow in calf starters resulted in slightly lower apparent digestibility of dry matter, organic matter, crude protein and nitrogen-free extractives, but that crude fibre was unaffected. However, Esplin *et al* (1963) found that addition of 4 per cent of tallow to a high concentrate fattening ration for 830 lb Hereford steers was associated with slight but not significant increases in the digestibility of dry matter, crude protein and crude fibre, and a slight decrease for nitrogen-free extractives. The present results indicate that in the absence of roughage the digestibility of organic matter was slightly higher on the diets containing tallow and palm-kernel oil, but no improvement was evident when hay was being fed. In both parts of the experiment the digestibilities of crude protein and total lipid were higher on the high-fat treatments, but only in the latter case did the increases attain significance. In both the absence and the presence of hay the digestibility of crude fibre and nitrogen-free extractives appeared to be unaffected by addition of fat.

In addition to palatability and digestibility considerations it is also necessary to consider the function of absorbed nutrients in order to fully evaluate the nutritional value of fat in concentrate mixtures for calves. The growing calf requires nutrients to meet its needs for both maintenance and growth, and in considering the optimum balance of nutrients to meet these requirements it is useful to consider the chemical composition of body gain. Mitchell (1962) calculated the gains in empty body weight by 150 lb Holstein calves to contain 8.4 per cent fat, 19.0 per cent protein and 67.1 per cent water. As the animal body contains relatively little carbohydrate it is evident that fat and protein account for a very high proportion of the total dry matter of body gain. In considering energy expenditure associated with the formation of body fat and protein it is evident that the retention of absorbed amino acids is potentially an efficient process because if protein could be supplied with a biological value of 100 per cent the only additional energy required would be that needed to synthesize the peptide bonds which link amino acids together in proteins (Blaxter 1967). In practice, however, the biological value of amino acids absorbed from the digestive tract by cattle is much lower (mean value about 70 per cent), and considerable synthesis of non-essential amino acids from various carbon fragments probably occurs. The efficiency of utilisation of absorbed amino acids is also influenced by other factors, notably the energy intake of the animal. Amino acids not used for maintenance and the synthesis of body protein can serve as a source of energy for fat synthesis, but when used in this way energy is required to synthesise urea and for the conversion of carbon fragments to fat, both of which lead to lower efficiency.

In the case of dietary fat, although it undergoes lipolysis in the rumen, and glycerol is readily fermented to volatile fatty acids, very little degradation of long-chain fatty acids occurs. The subsequent intestinal absorption of these fatty acids should permit a much higher efficiency of synthesis of body fat, from an energy standpoint, than is obtainable with steam-volatile fatty acids. Some indication of the efficiency of utilisation of the energy of long-chain fatty acids by ruminants has been provided by Czerkawski, Blaxter and Wainman (1966). In experiments with sheep they obtained efficiencies of utilisation for energy retention of 74.6, 79.2 and 82.5 per cent for the metabolisable energy of oleic, linoleic and linolenic acids respectively. These results suggest that long-chain fatty acids, once absorbed, may largely undergo direct incorporation into body fat. It is evident, therefore, that the inclusion of acceptable levels of fat in concentrate mixtures for ruminants is likely to improve their energy value in a number of ways. Firstly, as is evident in the present experiment, through substitution of fat, with its high calorific value per unit weight, for carbohydrate. A further increase in energy value occurred on the palm-kernel oil treatments because the added fat had a slightly higher digestibility than that of the nitrogen-free extractives it displaced. Finally, since long-chain fatty acids do not undergo fermentation in the rumen they avoid losses of energy in the form of waste gases and heat of fermentation, and when absorbed can be used with greater efficiency for the synthesis of body fat than can steam-volatile fatty acids resulting from the fermentation of carbohydrates. The improvements in energy value obtained through the inclusion of tallow and palm-kernel oil in the present experiment are borne out by the finding that their use led

to improvements in efficiency of retention of nitrogen. In addition to the fact that this represents more efficient use of dietary protein its use for this purpose is also likely to effect some improvement in overall efficiency of energy utilization.

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Methyl Esters of Tallow Fatty Acids in a Poultry Ration

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In two trials with Indian River Cross chicks 3, 5, and 7 and 7, 11, and 15% of No. 2 tallow methyl esters were substituted for milo in a practical soybean meal chick ration. Control groups were fed 7, 11, and 15% of the tallow from which the esters were prepared. The rations with 7, 11, and 15% lipide also contained 0.3% methionine. One 15% methyl ester group without methionine was added as a control. It was found that 3 to 15% of methyl esters are not toxic to poultry as measured by the rates and efficiencies of gain. During the first 5 weeks, at least, methyl esters and tallow increase the feed efficiency in proportion to the increased caloric value of the rations. Neither influences rate of growth. On high-fat rations added methionine increases the growth rate and feed efficiency of female but not male chicks, at least through 6 weeks of age. Methyl esters mix quickly, easily, and smoothly with a dry ration.

The production of inedible fats and oils has exceeded the demand (*1, 2*), during the past few years. The price, therefore, has been reduced to the level where these products can compete with corn and other sources of energy, in the manufacture of feeds for livestock.

For the most part, studies on the utilization of fats added to animal feeds have been confined to the better grades of tallow (*3, 5, 6*). To take full advantage of the price structure, however, it would be desirable to use the lower grades of tallows and greases (*4, 7*) or even acidulated soap stock.

The higher grades of tallow have several disadvantages. Because its relatively high melting point causes the tallow to solidify in tanks and pipes in cold weather, and it is often difficult to mix it without lumpiness, special equipment for adding fat to animal feeds is almost essential.

A possible solution to some of these problems is to convert inexpensive fats of high free fatty acid content to their methyl or ethyl esters. The esters, having a lower melting point than either the corresponding fatty acids or glycerides are easier to handle. Because of their lower viscosity and surface tension, they mix very readily, spread thinner, and keep dust down in a feed at a lower level than either fatty acids or glycerides.

Ethyl esters have a lower melting point than methyl esters, and the alcohol is known to be nontoxic. On the other hand, dry ethanol is considerably more expensive than dry methanol, its use requires government supervision, and it cannot be recycled for ester formation after it has been diluted with water, because it forms an azeotrope. Methanol does not form an azeotrope with water and may readily be distilled from water and recycled.

A survey of the literature revealed little information on the level at which methyl esters may be fed to farm animals without toxic symptoms, and no comparisons appear to have been published on the relative effects of esters and glycerides on growth and feed efficiency.

Table I. Basal Ration of Chicks Fed Methyl Esters and Tallow

Constituent	%
Soybean oil meal	35.0
Ground yellow corn	30.0
Ground milo[a]	28.0
Alfalfa leaf meal	3.0
Dicalcium phosphate	2.0
Ground oyster shell	1.5
Salt	0.5
Manganese sulfate, g./100 lb.	8.0
Methionine[b]	0.3
Vitamins and Antibiotics Added per Pound of Feed	
Riboflavin, mg.	2.0
Pantothenic acid, mg.	5.0
Niacin, mg.	12.5
Choline, mg.	400.0
Penicillin, mg.	1.0
Bacitracin, mg.	2.5
Vitamin A, I.U.	2270.0
Vitamin D, I.U.	600.0
Vitamin B_{12}, γ	3.0

[a] Methyl esters and tallow substituted for milo, pound for pound.
[b] Substituted for milo in second series of tests.

It was decided, therefore, to test the toxicity, growth, and feed efficiency factors of methyl esters prepared from No. 2 tallow at 3, 5, 7, 11, and 15% of the diet, and to compare the esters to tallow at the 7, 11, and 15% levels.

Experimental

Rations. The basal ration is given in Table I. Tallow methyl esters and tallow were substituted for milo pound for pound. Because of the increase in caloric value of the rations containing higher levels of fatty material, it was considered possible that the daily intake of methionine might be suboptimum. Therefore, 0.3% methionine was added to the high-fat rations. A group receiving 15% esters without added methionine was used as a test for this factor.

Procedure. Two series of tests were made. In the first series, four groups each of 25 unsexed day-old Indian River Cross chicks received the basal ration and 3, 5, and 7% of methyl esters, respectively. They were vaccinated against New Castle disease and placed in electrically heated batteries with raised wire floors. All birds received the basal ration for the first week. At the end of that period, they were wing banded and placed on the experimental diets for 9 weeks. The birds were weighed weekly and feed consumption was determined weekly except for the fifth and seventh weeks of the test. After the fourth week the weights of the males and females were recorded separately, and the average weights calculated as weighted averages.

In the second series of tests 7, 11, and 15% esters were fed, and as controls 7, 11, and 15% of the tallow from which the esters were prepared. Because of the

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high fat level, 0.3% methionine was added to these rations. As a control, one additional group was fed the 15% ester ration without 0.3% methionine. Eleven males and 11 females were placed on each ration, and feed efficiencies of each sex were recorded separately.

Results

The results of the first series of tests are recorded in Table II. During the first 4 weeks of the test the average weights of the birds in each group were recorded. For the last 4 weeks the two sexes were weighed separately and the weighted average weights calculated.

There was no significant difference in the average weights of the chicks at 10 weeks of age. Although the level of significance of the differences in feed efficiency may not be great the ratios of the weights of feed consumed to gains in weight are constantly lower in the ester-fed groups than in the basal. Furthermore, the higher the ester level, the lower the ratio.

There is no indication of toxicity in methyl esters.

The average gain in weight, total weight, and feed efficiency of the chicks in the second series of tests are given in Table III. After 5 weeks of test, at 6 weeks of age, with the exception of the chicks on 15% tallow, the gain in weight increases with increases of fatty material in the diet. Without exception, the feed efficiency improves with increase in fatty material in the diet. Roughly, the percentage of improvement in feed efficiency is the same as the increase in caloric value of the rations. Neither esters nor tallow have any influence on rate of growth.

The addition of methionine appears to have given some advantage to the females receiving it, but to have no influence on males. Methyl esters show no toxicity, since at the 15% level the chicks receiving the esters did better than those on tallow.

Unfortunately, during the last 4 weeks of the 9-week test the birds were disturbed by unusual activity in the animal quarters. The average weight was less than 1100 grams, while in the first series it approached 1300 grams.

Although small percentage differences have been lost, the results show that tallow has no advantage over methyl esters, and that the latter are not toxic at 15% of the ration for 10 weeks.

Subjectively it was found that methyl esters mix quickly and evenly into a dry ration and require no special equipment. Although the minimum amount of esters which would be required to prevent dustiness, as compared to tallow was not determined, the dust was laid with smaller amounts and more quickly by the esters.

Table II. Weights and Feed Efficiencies of Indian River Cross Chicks on Methyl Ester Rations

(Test Series I)

				Weeks of Age						
				2	3	4	5	7	9	10
				Weeks of Test						
Ration	Males	Females	Measured	1	2	3	4	6	8	9
Basal	17	13	Av. wt.[a]	170	261	343	513	823	1099	1288
			Feed efficiency	1.87	2.00	2.36	2.31	2.53	2.71	2.78
3% esters	12	18	Av. wt.	164	255	336	498	808	1109	1278
			Feed efficiency	1.69	1.80	2.17	2.74	2.43	2.55	2.68
5% esters	14	16	Av. wt.	161	253	348	501	834	1105	1285
			Feed efficiency	1.89	1.99	2.28	2.28	2.38	2.58	2.66
7% esters	18	12	Av. wt.	171	274	353	539	858	1118	1278
			Feed efficiency	1.88	1.92	2.29	2.17	2.32	2.52	2.63

[a] For first 4 weeks on test only average weights are given. For the last three weighings (fifth through ninth weeks) males and females were weighed separately and weight average weights for two sexes calculated.

Table III. Weight and Feed Efficiency

Diet	Av. Wt. Gain, Grams			Av. Total Wt., Grams			Feed Efficiency[b]		
	Male	Female	Av.	Male	Female	Av.	Male	Female	Av.
			Fifth Week on Test[a]						
Basal	589	469	504	649	560	605	2.39	2.53	2.46
Methyl esters									
7%	572	446	509	684	534	609	2.24	2.39	2.32
11%	595	451	523	704	533	619	2.19	2.25	2.22
15%	554	497	526	654	584	619	2.12	2.14	2.13
15%, no added methionine	554	415	485	663	498	581	2.16	2.54	2.35
Tallow									
7%	527	471	499	635	560	598	2.28	2.37	2.33
11%	620	478	549	726	568	647	2.14	2.32	2.23
15%	585	457	521	696	545	622	2.16	2.24	2.20
			Ninth Week on Test[c]						
Basal	1218	1115	1167	1108	1024	1066	2.73	2.68	2.69
Methyl esters									
7%	1368	1156	1262	1256	1068	1162	2.49	2.68	2.59
11%	1390	1027	1209	1281	945	1113	2.57	2.80	2.69
15%	1247	1094	1171	1147	1007	1077	2.64	2.54	2.59
15%, no added methionine	1279	1010	1145	1170	927	1049	2.57	2.64	2.61
Tallow									
7%	1232	1065	1149	1124	976	1050	2.68	2.63	2.66
11%	1401	1112	1257	1295	1022	1159	2.60	2.45	2.53
15%	1283	1104	1194	1172	1016	1094	2.65	2.41	2.53

[a] Birds 6 weeks old.
[b] Feed consumed/gain in weight.
[c] Birds 10 weeks old.

Conclusions

Methyl esters are not toxic to poultry when included from 3 to 15% in their rations, as determined by their effect on rate and efficiency of gain.

During the first 5 weeks, at least, methyl esters and tallow increase the feed efficiency proportionally to the increased caloric value of the rations.

Neither esters nor tallow have any influence on rate of growth.

On high-fat rations added methionine may increase the growth rate and feed efficiency of female but not male chicks, at least through 6 weeks of age.

Methyl esters mix quickly, easily, and smoothly with a dry ration.

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THROMBOGENICITY AND ATHEROGENICITY OF DIETARY FATTY ACIDS IN RAT

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SUMMARY

Among the saturated and unsaturated fatty acids studied, stearic and palmitic acids appeared to be the most thrombogenic when included in the hypercholesterolemic diet of rats, since they markedly predisposed the animals for the production of large, occlusive, endotoxin initiated thrombosis. Stearic and palmitic acids were also the most atherogenic, since fatty streaks could consistently be observed in the aorta of rats fed diets containing an excess of these fatty acids. Linoleic acid, a non-thrombogenic fatty acid, was the least atherogenic of the present series.

When purified triglycerides constituted the source of fat, tristearin and triolein were equally thrombogenic, although tristearin was much more atherogenic. Tricaproin was neither thrombogenic nor atherogenic.

Among the saturated fatty acids used, palmitic acid was the most and caprylic acid (or tricaprylin) the least hyperlipemic, a finding similar to that reported in man. Oleic acid, particularly in its triolein form, did not seem to be hypocholesterolemic. Of the blood tests carried out in the present experiment, none of them, *per se*, appeared to serve as a perfect indicator of the thrombotic tendency or the degree of atherosclerosis to be expected.

INTRODUCTION

It has been known for some time that dietary fats affect the serum lipid level in man[1]. This observation has been confirmed by numerous studies (refs. 2-4, to cite only a few) and has been thought to depend upon the proportion of unsaturated and saturated fatty acids in the fat[2]. Subsequently, it was reported that among the unsaturated fatty acids, only the polyenes could lower the serum cholesterol concentration[5,6] and, finally, even the ratio of polyunsaturated to saturated fatty acids had to be discarded as a possible means of predicting lipemia[7]. More recently it was shown in man that among the dietary saturated fatty acids, only lauric, myristic and palmitic acids, but not stearic acid, seemed to promote hypercholesterolemias[8].

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The question now arises whether the well known effect of dietary fats on the production of thrombosis and atherosclerosis[9] is also due to the most hypercholesterolemic fatty acids. It has been shown that the fatty acids with the longest chain, and particularly stearic acid, are also the most effective in aggregating blood platelets *in vitro*[10] and in inducing the formation of thrombi *in vivo*[12].

Recently, we showed that the thrombogenicity of a dietary fat appeared to be due to the type of fatty acid it contained rather than to its degree of saturation when multiple, large occlusive thromboses, mostly located in the hepatic veins, were initiated by the intravenous injection of a Gram negative bacteria lipopolysaccharide[13]. In these short term experiments, in which free saturated fatty acids or their esters were added to the diet, we observed that palmitic and stearic acids were the most thrombogenic fatty acids. In preliminary experiments[14], it seemed that the most thrombogenic fatty acids were also the most atherogenic, when saturated and unsaturated fatty acids were added at a 3 % level to the basic butter diet of albino rats. However, in an attempt to obtain more clearcut results, experiments of a similar type were undertaken in hooded rats, a strain that we have found to be more susceptible to atherosclerosis than albino rat, under certain conditions[15], and the level of the fatty acid added to the diet was increased to 6 %. In addition, serum cholesterol, triglycerides and lipoproteins, and also the plasma clotting time were determined at various periods, in order to more accurately assess the value of these determinations in predicting the thrombotic tendency or the degree of the atherosclerotic lesions.

Since it has been reported that the coefficient of digestibility of a fatty acid varies according to whether the free or the esterified form is ingested[16,17], verification of the results obtained with free fatty acids was made in Holtzman rats, this time utilizing purified triglycerides as the sole source of fat. The present communication describes the results obtained.

MATERIAL AND METHODS

This study constitutes the result of several series of experiments, with 6 animal per group. The results represented the pooled data of two sets of experiments in the first part of series 1 (Table 1) and in series 2 (Table 2), and three sets in series 3 (Table 3). The exact number of animals that survived until the time of sacrifice listed in the corresponding table. Male hooded rats of the Quebec Breeding Farm (St-Eustache, P. Qué., Canada) (series 1 and 2) and male albino rats of the Holtzman Co. (series 3), with an initial body weight of 140–170 g, were utilized for this study. The rats were housed in a constant temperature environment and given tap water and the high fat diet, *ad libitum*. For series 1 and 2, in addition to the fatty acid listed in the corresponding tables and included in the diet at the 6 %, level, the diets were composed of: butter 32, casein 11, cellulose 15, cholesterol 5, salt mixture 5, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight %)* as reported in detail elsewhere[13]. The experiments were terminated after 9 weeks (part 1 of series 1

Table 1), 15 weeks (part 2, Table 1) or 16 weeks (series 2, Table 2) of the dietary feeding. In series 3 (Table 3), purified triglycerides were utilized to replace butter and fatty acids. Gas chromatography of the triolein* and the tristearin** gave the following results. Triolein: oleate 68.3 %, linoleate 9.5 %, palmitoleate 8.1 %, palmitate 6.7 %, myristate 4.1 %, stearate 1.6 %. Tristearin: stearate 86 %, palmitate 11.9 %, laurate 1.3 %, and myristate 0.8 %. Tricaprylin was reported by the manufacturer** to be 95 % pure.

The consistency of the diet when tristearin alone was included at a 32 % level was markedly different from the consistency of diets with triolein or tricaprylin at the same level. As a corrective measure, a small amount (6 %) of triolein was substituted at the expense of these two triglycerides in the corresponding diets (Table 3). In addition, it was found in preliminary experiments that 5 % cholesterol and 2 % sodium cholate were too harmful to the animals when these triglycerides were utilized instead of butter and fatty acids. For this reason, the concentration of cholesterol was decreased to 1 % and that of sodium cholate to 0.5 %. Therefore, the composition of the diets, in addition to the triglycerides listed in Table 3, was as follows: casein 11, cellulose 16.5, cholesterol 1, linoleic acid 1, salt mixture 4, sodium cholate 0.5, sucrose 33, vitamin mixture 2 (weight %)*. Part 1 of this series 3 was terminated at 11 weeks and part 2 at 20 weeks. At the end of the feeding periods and following 17 h fasting, approximately 3 ml of blood were removed in 2 ml siliconized syringes, using 20-gauge needles, from the jugular vein of the rats, under ether anesthesia, as reported in detail elsewhere[13]. In part 1, series 1 (Table 1) and 3 (Table 3), a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide (0.6 mg/kg series 1, 1 mg/kg series 3) in saline was injected by the same needle, after withdrawal of blood.

In each rat, the plasma clotting time after recalcification was performed in triplicate, in plastic tubes (cellulose nitrate, No. 654, or polycarbonate No. 2804, International Equipment Co., Boston), according to the technique reported previously[13]. The lipemic parameters were determined on individual sera in series 3, but on pooled sera in series 1 and 2 in such a way that 3 (8 week period, series 2) or 6 (series 1 and 2) determinations per group were performed. The total serum cholesterol was determined by a Technicon autoanalyzer, using the technique recommended by the manufacturer; the triglycerides, by the VAN HANDEL method[18]; the lipoproteins by paper electrophoresis post-stained with fat-red 7B according to our modification[13] of the STRAUS AND WURM technique[19]. Only the percentage of the $\alpha_2 + \beta$ lipoproteins are reported here in Table 2, since in this series the other fractions did not give a more precise indication of the severity of the thrombotic and atherosclerotic lesions. Following the *S. typhosa* lipopolysaccharide injection (experiment 1, part 1), most of the animals died in 3 to 18 h; the survivors were killed 24 h after the injection.

* The salt mixture (Wesson), the vitamin mixture (Vitamin Diet Fortification Mixture), the cellulose (Alphacel), the fatty acids and the triolein utilized in all these experiments were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio (U.S.A.).
** The tristearin and tricaprylin was kindly supplied by the Drew Chemical Co., Boonton, N.J. (U.S.A.).

Autopsy was performed on all animals and the red hepatic infarcts were evaluated macroscopically. Previous sequential studies showed that the red hepatic infarcts are the result of occlusive thromboses of the hepatic veins[20]. The number and severity of the infarcts appear to correspond exactly to the number and the size of the thromboses. In addition, at the dosage used here, and provided no other treatment is given[21], occlusive thromboses are not usually found in organs other than the liver after *S. typhosa* lipopolysaccharide injection. Therefore, evaluation of the severity of thrombosis can be made simply by macroscopic evaluation of the number and extent of the hepatic infarcts, as already reported[20]. The gradation utilized was the following: 0, no infarct; 1, one to three infarcts up to 1 cm² each; 2, one to three infarcts up to 2 cm² each; 3, large infarcts of several lobes.

For assessment of atherosclerosis, the animals were killed with chloroform and the lesions evaluated under binocular examination of the aorta, opened longitudinally from the aortic valve to the iliac arteries. The gradation was as follows: 0, no lesions; 1, one to several yellowish white, raised fatty streaks; 2, involving a quarter of the intimal surface; 3, involving half or more of the intimal surface.

The macroscopic reading of both thrombosis and atherosclerosis was verified by histologic examination of the tissue concerned.

RESULTS

Series 1 (Table 1)

After 9 weeks of the dietary feeding (part 1), the endotoxin injection induced a high mortality in every group, but thrombosis resulted in groups 4, 5 and 6, only the highest severity being noted in group 5 fed stearic acid; this group also presented the highest cholesterol and triglyceride levels. However, in group 1, in which the lipemia was the next highest, no thrombosis could be detected. Nor could any relationship be found between the severity of thrombosis and the plasma clotting time, since the longest time was seen in group 5, which was fed stearic acid and presented the most severe lesions, and the lowest was in group 3, which presented no lesions. Part 2 of this experiment served as a pilot study for experiment 2, with regard to the development of atherosclerosis after 15 weeks of dietary feeding. The lesions observed here consisted of fatty streaks, with one or several layers of foam cells covered by endothelial cells, but little fibrosis. They are most conspicuous in the abdominal aorta and on, or in the vicinity of, the aortic valves. In the most severe cases, the lesions are encountered all along the aorta. As shown in Table 1, they occurred with the highest severity in group 5 fed stearic acid, the group in which the lowest clotting time was recorded, after 15 weeks of dietary feeding. The least severe lesions were observed in groups 2 and 3 fed linoleic and linolenic acids, respectively. In group 6, fed behenic acid, and exclusively in this group, we consistently observed lesions of a much more advanced type, closely resembling the human atheromatous plaque and mostly located in the valsalva sinus. These lesions were

TABLE 4

INFLUENCE OF DIETARY FATTY ACIDS ON LIPEMIA, CLOTTING TIME, THROMBOSIS, AND ATHEROSCLEROSIS IN INBRED RAT

Results: mean ± S.E. Duration of the experiments: part 1, 9 weeks; part 2, 15 weeks.

	Fatty acid (carbon atoms)	*Oleic 18 : 1*	*Linoleic 18 : 2*	*Linolenic 18 : 3*	*Caprylic 8 : 0*	*Stearic 18 : 0*	*Behenic 22 : 0*
	Group	*1*	*2*	*3*	*4*	*5*	*6*
Part 1	Number of animals	10	12	12	11	12	12
	Plasma clotting time (sec)	137 ± 12	169 ± 7	135 ± 5	149 ± 5	173 ± 5	168 ± 10
	Serum cholesterol (mg/100 ml)	316 ± 40	202 ± 16	201 ± 32	236 ± 22	391 ± 34	219 ± 10
	Serum triglycerides (mg/100 ml)	69 ± 12	33 ± 6	24 ± 6	43 ± 10	96 ± 11	35 ± 7
	Thrombosis severity (0–3)	0	0	0	1.1	2.7	1.1
	Mortality (%)	80	50	83	45	91	50
Part 2	Number of animals	6	6	6	6	5	6
	Plasma clotting time (sec)	167 ± 22	178 ± 12	143 ± 8	137 ± 11	128 ± 9	164 ± 18
	Atherosclerosis severity (0–3)	0.8	0	0.1	0.5	1.1	0.8

TABLE 2

INFLUENCE OF DIETARY FATTY ACIDS ON LIPEMIA, CLOTTING TIME, AND ATHEROSCLEROSIS, IN HOODED RAT

Result mean ± S.E. Total duration of the experiment 16 weeks.

Fatty acid (carbon atoms)		*Oleic 18 : 1*	*Linoleic 18 : 2*	*Linolenic 18 : 3*	*Caprylic 8 : 0*	*Palmitic 16 : 0*	*Stearic 18 : 0*	*Behenic 22 : 0*
Group		*1*	*2*	*3*	*4*	*5*	*6*	*7*
	Weeks							
Number of animals		11	13	9	11	6	8	12
Plasma clotting time (sec)	8	175 ± 10	177 ± 7	200 ± 24	170 ± 13	156 ± 7	176 ± 6	217 ± 9
	12	200 ± 30	186 ± 9	151 ± 11	162 ± 10	148 ± 9	162 ± 8	164 ± 7
	16	171 ± 15	171 ± 11	150 ± 10	142 ± 14	120 ± 8	126 ± 9	153 ± 7
Serum cholesterol (mg/100 ml)	8	241	197	187	233	758	391	234
	12	440 ± 43	296 ± 21	538 ± 73	370 ± 67	1075 ± 66	638 ± 120	311 ± 18
	16	655 ± 92	493 ± 47	1371 ± 154	363 ± 41	1530 ± 256	648 ± 54	290 ± 8
Serum triglycerides (mg/100 ml)	8	59	42	28	76	139	96	43
	12	84 ± 6	92 ± 11	128 ± 25	89 ± 19	176 ± 7	108 ± 10	90 ± 13
	16	91 ± 5	79 ± 6	257 ± 8	107 ± 9	270 ± 26	163 ± 25	71 ± 3
Serum lipoproteins (percentage α_2 ... β)	8	54	53	53	54	59	62	54
	12	58 ± 1.4	54 ± 1.4	58 ± 1.9	54 ± 1.6	61 ± 2.6	60 ± 1.6	52 ± 1.7
	16	58 ± 2.3	57 ± 1.6	67 ± 3.2	56 ± 1.5	64 ± 2.8	59 ± 1.7	51 ± 1.5
Aortic atherosclerosis (Scale 0–3)								
thoracic		0.7	0	0.2	1.1	2.0	2.0	0.5
abdominal		0.5	0	0.3	0.6	1.0	1.9	0.5
valve		0.6	0	1.3	0.3	1.3	1.0	0.9
mean		0.6	0	0.6	0.7	1.4	1.6	0.6

Fig. 1. On the left, a fibrous plaque with lipid material mostly located near the internal elastic membrane (arrows) on the wall of the valsalva sinus of a rat fed behenic acid. On the right, a fatty streak at the orifice of a coronary artery in a rat fed stearic acid. Here the lesion consists almost solely of foam cells with disruption of the elastic membrane. Hematoxylin phloxine saffron, ×220.

raised and consisted of numerous foam cells and cholesterol crystals lying on the intimal surface and abundantly covered by fibrosis (Fig. 1).

Series 2 *(Table 2)*

In this experiment, the cholesterol, triglycerides and lipoproteins in serum, and the plasma clotting time were analyzed after 8, 12 and 16 weeks of dietary feeding. At all three periods, group 5 fed palmitic acid presented the highest cholesterol, triglyceride and lipoprotein levels of all the groups. Nevertheless, the lesions did not appear to be more severe than those in group 6 fed stearic acid and in which the cholesterol and triglyceride level was much lower. However, there was no significant difference in the percentage of α_2 + β lipoproteins between groups 5 and 6 at the three periods or in the clotting times at the 16-week period. After 8 weeks, groups 2 and 3 fed linoleic and linolenic acids, respectively, presented the lowest lipemia. However, in these two groups, and particularly in group 3, a marked increase in the lipemia was noted at the 12- and the 16-week periods. Only in group 2 fed linoleic acid were no lesions recorded, while in group 3, the severity of the fatty streaks was comparable to those of groups 1, 4 and 7. In most of the animals of group 7 fed behenic acid, the peculiar atheromatous plaques described in experiment 1 were also observed, still mostly located in the valsalva sinus.

Series 3 (Table 3)

After 11 weeks of dietary feeding (part 1) there was no significant difference in the plasma clotting time or in the serum cholesterol between groups 1, 2 and 3. However, the rats of group 2 fed tricaprylin presented significantly lower triglycerides than those of group 1 fed triolein, while the animals of group 3 fed tristearin exhibited significantly higher triglycerides than the other groups. The mortality rate was lower in group 1 than in group 3 and was the lowest in group 2. No thrombosis was recorded in group 2, while the severity of the lesions was the same in group 1 and 3.

After 20 weeks of dietary feeding (part 2) the plasma clotting time was the longest in group 2, while there was practically no difference between group 1 and 3. The serum triglycerides were still the lowest in group 2, but no significant difference was noted between group 1 and 3. With regard to atherosclerosis, a severity of 1.9 was registered in group 3 as compared with 0.6 in group 1 and 0.1 in group 2.

DISCUSSION

It has been reported that, in the normal rabbit[22], dog[23] and rat[24], one intravenous injection of a Gram-negative bacteria lipopolysaccharide is able to induce shock with a high mortality rate. At autopsy, microthrombi can be detected in several organs and particularly in the hepatic sinusoids. In contrast to this, in rats fed certain high fat diets, the intravenous injection of a lipopolysaccharide will initiate the formation of huge occlusive thromboses in the large hepatic veins[20,21,25]. Since the enormous increase in the thrombus size is apparently due to the high fat diet, the diet can be

TABLE 3

THROMBOGENICITY AND ATHEROGENICITY OF DIETARY PURIFIED TRIGLYCERIDES

Results = mean ± S.E. Diet composition in addition to the triglyceride: casein 14, cellulose 2, cholesterol 1, linoleic acid 1, salt mixture 4, sodium cholate 0.5, sucrose 33, vitamin mixture. Duration of the experiment: part 1, 11 weeks; part 2, 20 weeks. Group comparison. Triglycerides: part 1, group 1 vs. 2 and 3, $P < 0.01$, 2 vs. 3, $P < 0.001$; part 2, group 2 vs. 1 and 3, $P < 0.001$. Plasma clotting time, part 2, group 2 vs. 1, $P < 0.01$ and 3 $P < 0.001$.

		Triolein 31	*Tricaprylin 25 + triolein 6*	*Tristearin 25 + triolein 6*
	Group	*1*	*2*	*3*
Part 1	Number of animals	14	16	16
	Plasma clotting time (sec)	192 ± 9	190 ± 11	182 ± 8
	Cholesterol (mg/100 ml)	617 ± 69	777 ± 74	770 ± 106
	Triglycerides (mg/100 ml)	178 ± 9	123 ± 10	250 ± 22
	Thrombosis-severity (0–3)	0.8	0	0.8
	Mortality	36	0	50
Part 2	Number of animals	14	17	14
	Plasma clotting time (sec)	131 ± 5	188 ± 12	133 ± 6
	Cholesterol (mg/100 ml)	1433 ± 120	639 ± 102	999 ± 106
	Triglycerides (mg/100 ml)	214 ± 21	106 ± 6	233 ± 15
	Atherosclerosis-severity (0–3)	0.6 ± 0.4	0.1 ± 0.06	1.9 ± [illegible]

qualified as thrombogenic. This easily reproducible experimental model appears to be particularly suitable for studying which factor in the diet, and specifically in the fats, is responsible for its thrombogenicity.

Although more study is needed to verify this, under these experimental conditions we have repeatedly observed that the mortality rate of the animals was dependent not so much on the severity of the thrombotic phenomena but rather on the susceptibility to shock, which seems to be connected with the triglyceride level[25]. In the present study, a high mortality rate following endotoxin injection occurred in animals fed stearic and oleic acids (series 1), and in those fed tristearin and triolein (series 8); these animals also presented the highest serum triglycerides. Nevertheless, in the group fed linolenic acid, the mortality rate was also, unexplicably, extremely elevated, despite a low triglyceride level.

The present study in the hooded rat concerning the inclusion of free fatty acids in the diet confirms our preliminary work, namely, that the fatty acids found to be the most thrombogenic, *i.e.*, palmitic and stearic acids, also appear to be the most atherogenic[11]. However, when fatty acids were included at only a 3% level in the diet of albino rats, linoleic and linolenic acids were by far the most hypolipemic and the least atherogenic[14]. Under the present conditions, linoleic and linolenic acids were hypolipemic only for the first 8 weeks. Linoleic acid was still the least atherogenic, but the atherogenicity of linolenic acid was comparable to that of oleic, caprylic, and behenic acids. The variation between the present results and those previously reported[14] could be due to the differences in the strains of rats used or the level of the fatty acids included in the diet. Although more experiments are needed to verify this, it is probable that the dissimilarity is due to the different level of the polyunsaturated fatty acids. In man also, it has been observed that dietary inclusion of polyunsaturated fatty acids at too high a level will favorably influence serum cholesterol only for a limited period of time[25].

In general, the effect of the fatty acids on lipemia observed here in the rat appears to be comparable to that reported recently in man, namely, that palmitic acid is much more hyperlipemic than stearic acid and that saturated fatty acids of less than 12 carbon atoms (caprylic acid and tricaprylin, here, for example) do not seem to increase serum cholesterol[3]. Finally, it is also known that, in man, oleic acid has no lowering effect on the serum cholesterol[27], a result also observed here in the rat with the acid or the triglyceride.

Until now, it has been generally recognized that atherosclerotic lesions are predisposing factors for thrombosis and that thrombi may play a role in the formation of atherosclerotic plaques[28]. Nevertheless, certain epidemiologic studies have shown[6,29,30] that, in addition to atherosclerosis, thrombosis itself can be influenced by the dietary fats. The observations seem to be confirmed by the present experiments, which indicate that, in general, the most thrombogenic fatty acids may also be the most atherogenic. However, a discrepancy has been noted here between the experiments with fatty acids and those with triglycerides. Although tristearin seems to be much more atherogenic than triolein, tristearin and triolein were equally

thrombogenic. This is also confirmed by the fact that there was practically no difference between the clotting time of tristearin and of triolein-fed rats. It has been reported that the coefficient of digestibility of tristearin is inferior to that of the corresponding fatty acid, in the rat, while that of triolein is superior[15]. This could explain why triolein seems to be more thrombogenic than oleic acid, and tristearin less so than stearic acid; but it does not explain why tristearin was much more atherogenic than triolein. At any rate, since dietary fats are usually ingested under the form of triglycerides, it is the response obtained with these substances that has to be considered for practical applications. Nevertheless, oleic acid could be the only fatty acid able to induce a result differing markedly from that obtained by the corresponding triglyceride. In experiments designed to test common fats under the conditions used here, the thrombogenicity of the fats could be predicted from their composition in fatty acids, in the light of the results obtained with the fatty acids themselves, oleic acid being an exception. As an example, cacao butter, which contains mostly stearic and palmitic acids in addition to oleic acid, was by far the most thrombogenic fat (to be published elsewhere). The present experiment with the purified triglycerides also indicates that, provided thrombogenic and atherogenic fatty acids are utilized the cholesterol and the bile salt included in the diet could be markedly reduced without affecting the result. In this connection, it has been reported recently[21] that, in the rabbit, only cacao butter was atherogenic in the absence of cholesterol.

In the series 2, although determinations of several parameters were performed at the 8-, 12- and 16-week period, none of these determinations *per se* appeared to serve as a perfect indicator of the thrombotic tendency or the degree of atherosclerosis to be expected. The results suggest that, not only the degree or the type of the lipemic change, but also its duration, has to be considered. An example of this was given by the group fed linolenic acid. While only minor atherosclerotic lesions were recorded in this group, the cholesterol and triglyceride levels determined at the time of autopsy were extremely elevated. By contrast, it was in this group that these parameters were the lowest at the 8-week period, a finding that can explain the mildness of the lesions. However, in series 3, at both the 11- and the 20-week period, the serum triglycerides appeared to be related to the severity of atherosclerosis.

As regards the plasma clotting time, more experiments are needed to further evaluate this test as an indicator of thrombosis and atherosclerosis, since marked discrepancies were noted in the present study. The effect of fatty acids included in the diet was consonant with the results of experiments performed *in vitro*[10,12,22] namely, that it is the long-chain saturated fatty acids that are the most effective in aggregating blood platelets and promoting coagulation. Behenic acid, however, could prove to be an exception in that it did not appear to be highly thrombogenic not only in the present experiment but also in those previously reported[13,14]. This could be due to the fact that, in the rat, behenic acid seems to be very poorly absorbed[16]. Nevertheless, the small amount absorbed could have been sufficient to induce the consistent production of peculiar atheromatous plaques in the group fed this fatty acid, plaques that are probably similar to those described by oth

vestigators[11] in rats fed arachis oil, a fat that contains long-chain saturated fatty acids, such as behenic acid. Finally, among the unsaturated fatty acids, oleic acid, which has been reported to be quite effective in aggregating blood platelets *in vitro*[13], could also be highly thrombogenic when ingested in the form of triolein.

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THROMBOTIC, ATHEROSCLEROTIC AND LIPEMIC EFFECTS OF DIETARY FATS IN THE RAT

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A few years ago, Pickering[1] noted, "The great weakness of the lipid hypothesis is that it does not account for the thrombus." He did not consider as satisfying to a clinician that alterations in lipid metabolism could also predispose to thrombosis. Nevertheless, numerous studies have indicated a relationship between thrombosis and hyperlipemia,[2-4] as well as the importance of lipids in blood clotting[5]; in addition, there is a strong possibility that the long-chain saturated fatty acids of the dietary fat are responsible for thrombosis[6] being by far the most effective fatty acids in aggregating blood platelets *in vitro*.[7, 8]

In recent experiments in rats, we devised an experimental model[9] that results in a constant and extremely elevated incidence of easily observed large occlusive thromboses, provided the animals are fed certain high-fat diets for 6 to 10 weeks.[10] Thrombosis is then initiated by the intravenous injection of a Gram-negative bacterin endotoxin, resulting in multiple, red hepatic infarcts, and a simple quantitative evaluation of the phenomenon can be achieved merely by weighing the lesions, as will be described in this paper.

By adding fatty acids to the basic high-fat diet, we were able to show[11, 12] that, under these conditions, as *in vitro*, the long-chain fatty acids were once again the most thrombogenic and that stearic acid appeared to be the most active among them. However, the very-long-chain fatty acids, such as behenic acids, were not thrombogenic, probably because of very poor digestibility.[12, 13]

In these studies in rats, as in man,[14, 15] palmitic acid was the most hypercholesterolemic of the fatty acids used, whereas stearic acid or short-chain fatty acids such as caprylic acid were much less hyperlipemic. As was also shown in man,[16] only linoleic and linolenic acids, but not oleic acid, were hypolipemic.

Other investigators working on the relationship between the human diet and its lipemic effects[16] recently suggested that people wishing to avoid coronary disease should substitute cacao butter for butter in the diet, because the former is richer in stearic acid, a long-chain saturated fatty acid that is much less hypercholesterolemic than palmitic, myristic and lauric acids. Judging from the results of work with blood platelets *in vitro*, such recommendations could be disastrous for coronary patients.

Inasmuch as our previous findings with dietary fatty acids seemed to have established the essential link between lipemia in man and the work with

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blood platelets and coagulation *in vitro*,[12, 13] it was deemed necessary, because of the obvious implications, to make a detailed study under our experimental conditions of most of the common fats utilized in human nutrition. Our aims in these experiments were to verify: (1) whether the thrombogenic, atherogenic and lipemic effects of a dietary fat could be predicted from its composition in fatty acids; (2) to what extent the degree of lipemia as expressed by the serum cholesterol or triglyceride level could be a reliable indication of the thrombogenic or atherogenic capacity of a fat.

In addition, since we had not previously studied myristic and lauric acids, and a knowledge of their effect under our experimental conditions was essential for the present work on dietary fats, these fatty acids were studied here in a small experiment in comparison with caprylic, palmitic and stearic acids.

MATERIAL AND METHODS

Male Holtzman rats with an initial body weight of 150 to 170 gm were utilized. For each experiment, the exact number of animals actually utilized is shown in the corresponding table. The results of experiment 2 (table 2) and experiment 3 (table 4) represent the pooled data of several sets of experiments, with 6 animals per group in each set. These data were pooled because they were practically identical and resulted from experiments performed at approximately the same time and under comparable conditions. The rats were housed in a constant temperature environment and given, *ad libitum*, tap water and the hyperlipemic diet described in detail elsewhere.[10, 11] In the tables are listed the ingredients composing the diets utilized in the corresponding experiment. The casein, cellulose (Alphacel), cholesterol, salt mixture (Wesson), sodium cholate, sucrose and vitamin mixture (vitamin diet fortification mixture) were obtained mixed in the right proportion by the supplier. The fatty acids utilized in experiment 1 (table 1) were also purchased from the same source (Nutritional Biochemicals Corporation). The animal fats utilized were ground and then melted in a steam bath; a sample of each of these tallows was analyzed by gas-liquid chromatography and the results are reported in table 3. Except for the coconut oil (Matheson, Coleman and Bell) and cacao butter, kindly supplied by Fry-Cadbury Ltd., all the fats and oils were commercially available and were therefore purchased from local stores. When the diets were prepared, isocaloric amounts of the fat (melted when necessary) were carefully mixed with the basic ingredients and then kept under refrigeration for no more than 2 weeks.

At the end of the feeding periods and following 17 hr of fasting, approximately 3 ml of blood were removed in siliconized syringes, equipped with 20-gauge needles, from the jugular vein of the rats, under light ether anesthesia, as reported in detail elsewhere.[11] To initiate thrombosis in experiments 1, 2 and 3 (tables 1, 3 and 4), a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide (0.3 mg per kg) in saline was injected by the same needle, after the last withdrawal of blood.

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At the end of the feeding periods and following 17 hr of fasting, approximately 3 ml of blood were removed in siliconized syringes, equipped with 20-gauge needles, from the jugular vein of the rats, under light ether anesthesia as reported in detail elsewhere.[11] To initiate thrombosis in experiments 1 and 3 (tables 1, 3 and 4), a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide (0.3 mg per kg) in saline was injected by the same needle, after last withdrawal of blood.

In each rat of the first three experiments, the plasma clotting time after recalcification was performed in duplicate, in plastic tubes (polycarbonate No. 2804, International Equipment Company, Boston), according to the technique already reported.[11] Triglycerides and total cholesterol analysis were performed on individual sera. The triglycerides were determined by the Van Handel method.[17] In experiment 3 (table 4), Florisil (60 to 100 mesh, Fisher Scientific Company) was used, instead of zeolite as suggested by other investigators[18], but chloroform was still kept as the extracting fluid. Only 0.3 ml of serum was utilized for this determination. The total serum cholesterol was determined by a manual adaptation of the Technicon Auto-Analyzer automated ferric chloride technique,[19] which yielded remarkably reproducible results. In brief, 0.2 ml of serum was added to 4.8 ml of isopropanol, carefully agitated and, after standing for 15 min., centrifuged for 5 min. To 0.5 ml of the supernatant were first added 4 ml of a 0.05 per cent ferric chloride solution in 95 per cent acetic acid and then 3 ml of concentrated sulfuric acid. After 5 min. of centrifugation, the reading was made at 550 mμ against a blank.

On 6 rats in each group of experiment 3 (table 4), no serum determinations were carried out; instead, heparinized plasma was collected on ice to determine the fibrinogen, according to the method of Goodwin[20] utilizing only 0.5 ml of plasma, and the unesterified fatty acids by the technique of Itaya.[21]

In the thrombosis experiments, the time of death was recorded and the animals were autopsied. Survivals were killed at 24 hr. with chloroform. Except in experiment 2 (table 2), in which the lesions were graded according to a scale of 0 to 3 as described elsewhere,[14] a quantitative evaluation of the thrombotic phenomenon could be obtained by weighing the liver before and after removing the red infarcts, as shown in figure 1. The severity of the thrombotic phenomenon was then expressed as the percentage in weight of infarcted liver.

In the atherosclerosis experiment (table 5), the animals were killed with chloroform after the last blood removal and the heart and the aorta were dissected as far as the common iliac arteries. The severity of the aortic fatty streaks was then graded according to a scale of 0 to 3 as already described,[14] under binocular examination of the fresh aorta, opened longitudinally. Macroscopic readings were verified by routine histologic examination of the heart and aorta.

RESULTS

Experiment 1 (table 1). Depending on the fatty acid added to the already high fat diet, the results obtained differed markedly. Palmitic acid gave rise to by far the highest values for cholesterol and triglycerides; the next most hyperlipemic fatty acids were caprylic and myristic acids, followed by stearic acid; lauric acid was the least hyperlipemic. In group 5 fed stearic acid, the plasma clotting time was the shortest and the severity of thrombosis the highest; this was followed by the results for group 4 in which the clotting time was significantly longer and the thrombotic lesions were less severe, although not

TABLE 1

Experiment 1: thrombogenicity of various fatty acids

Results = mean ± S.E. Duration of the experiment, 6 weeks. Diet composition in addition to the fatty acid (5 per cent) listed below: butter 32, casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2, water 1 (weight per cent).

Group and Fatty Acid	No. of Carbon Atoms	No. of Animals	Cholesterol	Triglycerides	Plasma Clotting Time	Thrombosis (Severity)	Survival
			mg./100 ml.		sec.		hr.
1. Caprylic	8	7	720 ± 172	156 ± 21	116 ± 8	2.0 ± 1.1	9.3 ± 2.3
2. Lauric	12	6	401 ± 33	111 ± 20	108 ± 17	2.3 ± 1.6	12.7 ± 3.4
3. Myristic	14	7	738 ± 37	154 ± 16	184 ± 9	0	7.4 ± 2.8
4. Palmitic	16	7	1153 ± 135	258 ± 39	143 ± 8	8.2 ± 3.6	3.3 ± 1.5
5. Stearic	18	7	528 ± 71	130 ± 15	121 ± 3	14.5 ± 6.3	11.4 ± 2.5

significantly so. The thromboses in the groups fed caprylic, lauric and myristic acids were much less severe, but only in groups 2 and 3 was the clotting time prolonged. The survival time was shortest in group 4, where the highest lipemia was seen, and longest in groups 2 and 5, where the lowest cholesterol and triglyceride levels were observed.

Experiment 2 (table 2). From experiment 1, it could be concluded that stearic acid was the most thrombogenic of the fatty acids studied. Therefore, if these results were to have practical applications for dietary fats, the fats richest in stearic acid should be the most thrombogenic. Table 3 shows the composition of various common fats differing in the type of fatty acids they contain, particularly in the quantity of stearic acid. From this table it can be seen that cacao butter contains 35 per cent stearic acid, butter 14.6 per cent, lard 7.8 per cent and corn oil 3.4 per cent.

As is seen in table 2, after only 8 weeks of dietary feeding, the severity of thrombosis of the butter-fed animals was 0.6 and the mortality rate was 30 per cent. The animals fed cacao butter presented a shorter clotting time, a severity of thrombosis of 2.1, and a 100 per cent mortality rate. Here, however, with cacao butter as the only fat, cholesterol and triglycerides were higher than with butter. After 10 weeks of dietary feeding, the severity of thrombosis in group 3 fed butter was 1.9. The serum cholesterol and triglycerides were no higher than after 8 weeks, but the clotting time was considerably decreased from 185 to 139 seconds. In group 4 fed lard, the severity of thrombosis was only 0.8; the cholesterol and triglycerides were lower and the clotting time longer than in group 3. The mere fact of reducing the butter content to 32 per cent and substituting 6 per cent of corn oil (group 5) markedly prolonged the clotting time and reduced the lipemia; the severity of thrombosis was only 0.4. When corn oil was the only fat in the diet (group 6), the triglycerides but not the cholesterol, showed a further decrease as compared with group 5; the clotting time was prolonged and the severity of thrombosis decreased to 0.1.

TABLE 2

Experiment 2: thrombogenicity of various fats

Results = mean ± S.E. Diet composition in addition to the ingredients listed below: casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Group and Duration	No. of Animals	Cholesterol	Triglyceride	Plasma Clotting Time	Thrombosis (Severity, 0-5)	Mortality	Survival
		mg/100 ml		sec.		%	hr.
8 weeks							
1. Cacao butter 32 + water 6	23	830 ± 23	148 ± 6	155 ± 7	2.1	100	4.2 ± 0.7
2. Butter 38	12	585 ± 80	135 ± 4	185 ± 12	0.6	30	18.1 ± 2.3
10 weeks							
3. Butter	33	487 ± 45	134 ± 12	139 ± 4	1.9	59	13.9 ± 1.6
4. Lard 32 + water 6	12	439 ± 25	115 ± 6	148 ± 8	0.8	58	14.2 ± 2.7
5. Butter 32 + corn oil 6	24	340 ± 21	94 ± 9	164 ± 11	0.4	46	16.7 ± 1.8
6. Corn oil 32 + water 6	12	366 ± 1	73 ± 4	185 ± 7	0.1	16	23.0 ± 0.7

TABLE 3

Composition of certain fats in the commonest fatty acids

Selected values adapted from, "Fatty acids in animal and plant products," by V. R. Goddard and L. Goodall, Human Nutrition Research Division, United States Department of Agriculture, 1959.

Fatty Acids (Carbon Atoms)	Coconut Oil	Butter*	Cacao Butter	Bovine Tallow*	Lard	Chicken Tallow*	Horse Tallow*	Olive Oil	Arachis Oil	Corn Oil
Lauric (12)	45.4	1.1								
Myristic (14)	18.0	9.1		3.3		1.0	5.6			0.1
Palmitic (16)	19.5	28.6	24.4	24.9	32.2	24.6	29.6	9.2	8.2	8.3
Stearic (18)	2.3	14.6	35.0	16.9	7.8	6.9	4.9	2.0	6.2	3.4
Arachidic (20)	0.4							0.2	4.0	0.4
Lignoceric (24)									3.9	0.2
Palmitoleic (16:2H)	1.3	2.7		5.3		6.5	6.0			1.2
Oleic (18:2H)	7.1	33.0	28.2	48.3	48.0	45.3	31.3	83.1	52.9	30.0
Linoleic (18:4H)	0.4	2.4	2.4	3.3	11.0	15.7	8.2	3.9	24.7	54.1
Linolenic (18:6H)		1.4			0.6		15.0			0.6
Iodine index	7.8	33.4	37.0	48.3	61.0	79.3	82.7	84.4	90.1	126.7

* Determined in the laboratory of Professor J. M. Demers, Biological Sciences, University of Montreal.

Experiment 3 (table 4). In this study, the results of four different experiments that gave almost identical results were pooled. In table 4 are reported all the data obtained in these experiments; the different fats are listed in order of their decreasing thrombogenic capacity. Although all of the determinations were performed under rigid control conditions by the most reliable methods found to be compatible with the handling of a large number of samples by well-trained technicians, none of the parameters examined seem to have any connection with the severity of thrombosis, and only the survival time appears to be related to the triglyceride level. Figure 2 shows a correlation ($r = -0.81$)

TABLE 4

Experiment 3: thrombogenicity of various fats

Results = Mean ± S.E. Total duration of the experiment = 9 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water), casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Group and Fat	Iodine Index	No. of Animals	Cholesterol	Triglycerides	Unesterified Fatty Acids	Fibrinogen	Plasma Clotting Time	Thrombosis (Severity)	Survival
			mg/100 ml		μmol/cc	mg/100 ml	sec.		hr.
1. Butter	33.4	56	393 ± 93	192 ± 22	690 ± 20	380 ± 20	130 ± 2	10.2 ± 2.1	8.8 ± 1.0
2. Arachis oil	94.8	13	720 ± 50	78 ± 9			160 ± 7	9.4 ± 2.5	18.3 ± 1.7
3. Coconut oil	7.8	25	685 ± 37	125 ± 6	590 ± 50	340 ± 10	164 ± 7	4.8 ± 1.7	10.8 ± 1.8
4. Horse tallow	82.7	25	234 ± 20	48 ± 4	420 ± 10	420 ± 10	134 ± 6	1.8 ± 1.3	16.6 ± 1.7
5. Chicken tallow	79.3	28	459 ± 40	80 ± 8	400 ± 30	390 ± 10	141 ± 4	3.7 ± 0.8	16.7 ± 1.4
6. Olive oil	84.4	23	1022 ± 101	133 ± 13	370 ± 40	400 ± 20	144 ± 4	0.9 ± 0.4	11.1 ± 2.0
7. Bovine tallow	49.3	28	2046 ± 100	186 ± 19	160 ± 20	480 ± 10	165 ± 9	0.8 ± 0.1	9.3 ± 1.1

FIG. 1. *Left.* Macroscopic appearance of liver of rat fed high-fat diet, showing multiple red infarcts 3 to 12 hr. after the endotoxin injection. *Right.* A similar liver in which the infarcted areas have been removed. The difference in the weight of the liver before and after removal of the lesions gives a fair indication of the severity of the thrombotic phenomenon.

on a group basis between the serum triglycerides and the survival time on the pooled data of experiments 1, 2 and 3. The survival time was also related to the serum cholesterol, but the correlation coefficient r was only -0.69.

Experiment 4 (table 5). As in experiment 2, the highest serum cholesterol triglyceride level at both the 10- and the 18-week period was observed in rats fed cacao butter; in these animals, the most severe atherosclerotic lesions were also noted. In contrast to this, the group fed olive oil presented, at both periods, a degree of lipemia comparable to the butter-fed rats, but the lesions were incomparably less severe in the rats fed olive oil. Corn oil, the fat with the lowest lipemic effect, was also the least atherogenic.

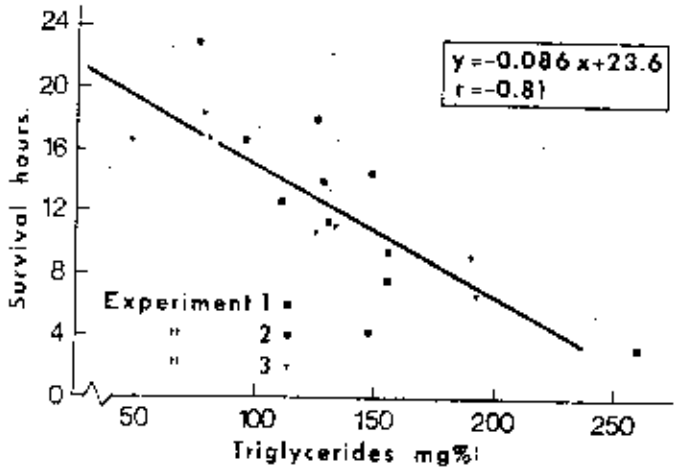

FIG. 2. The relationship, on a group basis, between the serum triglyceride level and the survival time in endotoxin-injected rats. The results are the pooled data of experiments 1, 2 and 3.

TABLE 5

Experiment 4: influence of dietary fats on lipemia and atherosclerosis

Results = Mean ± S.E. Total duration of the experiment 18 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water): casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Dietary Fat	No. of Autopsied Animals	Cholesterol		Triglycerides		Atherosclerosis (Scale 0–5)
		Week 10	Week 18	Week 10	Week 18	
		mg/100 ml		*mg/100 ml*		
Cacao butter	4	1136 ± 27	2775 ± 654	217 ± 14	582 ± 175	2.3
Butter	5	459 ± 11	2001 ± 696	124 ± 10	413 ± 160	1.7
Coconut oil	6	711 ± 9	516 ± 36	156 ± 25	117 ± 12	1.5
Olive oil	6	680 ± 203	1621 ± 342	188 ± 56	309 ± 70	0.3
Corn oil	6	301 ± 12	383 ± 34	57 ± 3	76 ± 9	0.05

DISCUSSION

The pathogenesis of our experimental model in which voluminous occlusive thromboses located almost exclusively in the hepatic veins are produced in rats fed a high-fat diet is not yet completely elucidated. Nevertheless, for years now, from one series of experiments to another, a continuously high incidence of multiple, red hepatic infarcts, resulting from the complete occlusion of the hepatic veins, has been consistently observed in rats fed the butter diet. We had already noted[9] that fatty infiltration of the liver has no bearing on the site of the thrombus formation. Despite the heavy fatty infiltration, it can also be added that liver protein synthesis in these animals is no different from that of rats fed laboratory chow.[22] In addition, we have observed in experiments not yet terminated that administration of epinephrine or norepinephrine instead of endotoxin to rats fed a high-fat diet will also initiate the formation of a voluminous thrombotic phenomenon, although in such cases the thrombi are located in the left atrium.

The most simple explanation for the hepatic veins as the primary site of thrombosis after an injection of Gram-negative bacteria endotoxin is that, in rat, the affinity of the liver for the bacterial toxins appears to be much greater than that of any other tissue.[23] It has also been observed[23] that, after the liver, the tissues retaining the largest quantity of toxins and the most frequent site of thrombosis are the spleen and the lungs. However, at the dosage of endotoxin utilized in this experiment, occlusive thrombi are found almost exclusively in the liver; therefore, from a practical point of view, only this organ has been considered in evaluating the severity of the thrombotic process.

The results of experiment 1 in this work confirm our previous results[12, 13] in that palmitic acid is much more hyperlipemic than stearic and caprylic acid, a finding that is also concordant with results obtained in man.[15, 16] In contrast to this, our observations with myristic and lauric acids are markedly different from those reported in man.[14, 16] Myristic acid induced a lipemia comparable to that induced by caprylic acid, and lauric acid was by far the least hyperlipemic of the five saturated fatty acids examined here. Of course, these results were obtained with purified free fatty acids and, as previously discussed,[13] the digestibility of the corresponding triglyceride can differ markedly from that of the acid and could explain the discrepancy in the results obtained when fats are used. However, among the fats studied in this series, only coconut oil contained large amounts of lauric and myristic acids. The results of experiment 3 showed that even in large-scale experiments, the cholesterol was the same in animals fed butter as in those fed coconut oil, and the triglycerides were lower in the latter group. Therefore, under our conditions, lauric and myristic acids cannot be highly hyperlipemic. From acute experiments with coconut oil in the dog,[24] it was concluded that saturated fatty acids of 12 and 14 carbon atoms had the most powerful effect in raising serum cholesterol, a finding that could also be applied to humans.[17]

However, the reason for this is far from clear, inasmuch as it has been reported that coconut oil decreases cholesterolemia in hyperlipemic patients[25] and that coconut oil fed to rabbits induced hypercholesterolemia only for 3 months,[26] an indication of the difficulties in drawing conclusions from short-term experiments. Under our conditions, the dietary feeding lasts for only 6 to 18 weeks, but in 6 to 10 weeks, owing to the type of diet, the animals are predisposed to the development of large thromboses that would normally occur in man, either in the coronary artery or in the veins,[27] only after 40 years of age. Therefore, in determining the value of our experimental model for reproducing human conditions, the results so obtained will have to be compared mainly with epidemiologic studies in man. As an example, it has been reported that the diet of the Atin-Mitiaro islanders[28] is high in coconut. The serum cholesterol of the 40- to 49-year-old men is 235 mg per 100 ml, a value certainly no higher than those observed in man in North America, the Netherlands and Finland, where butter and dairy products certainly represent the largest portion of the dietary fat.[29]

It is known that olive oil has no cholesterol-lowering effect,[14] and in our experiments with oleic acid its cholesterolemic effects were not much different from those of stearic acid.[12, 13] In animals fed olive oil as the sole fat, the resulting cholesterolemia was slightly higher than with butter. Bovine tallow, which is much richer in oleic acid than butter, yielded a similar result. In all the experiments presented here, the lowest lipemia was recorded after feeding horse tallow (experiment 3), corn oil (experiment 2) and chicken tallow, horse tallow being richer in linolenic acid, and the two others in linoleic acid. In previous experiments,[12] these two acids were found to be the least thrombogenic and may have even antagonized the damaging effect of stearic and palmitic acids. The marked reduction in thrombogenicity that resulted from substitution of 6 per cent of the butter by corn oil in experiment 2 also suggest an antagonizing effect of linoleic acid.

With regard to thrombosis, our aim was to verify whether dietary fats with a high content in stearic and palmitic acids, the acids found to be the most thrombogenic in previous experiments,[12, 13] would be also the most thrombogenic. In experiment 2, cacao butter, by far the fat richest in stearic acid, was in fact the most thrombogenic; corn oil, the poorest in this fatty acid, was the least thrombogenic. These results with fats confirm those obtained with free fatty acids in previous[12, 13] and this series, in that only palmitic and stearic acids appeared to be highly thrombogenic. These data are also concordant with studies *in vitro* indicating that the long-chain saturated fatty acids are the most effective in aggregating blood platelets. In addition, in this experiment, with a few selected fats markedly different in the type of fatty acids they contained, almost all of the parameters examined (cholesterol, triglycerides, clotting time) appeared to be related to the severity of thrombosis.

The atherosclerosis experiment also confirms our earlier results that the most thrombogenic fatty acids are also the most atherogenic, independently of their

lipemic effect. Olive oil, which is more hyperlipemic than coconut oil, appears to be much less atherogenic and also much less thrombogenic. We had already found that oleic acid was almost no more thrombogenic[18] than linoleic acid, and only slightly more atherogenic.[12, 13] However, we also found that purified triolein was as thrombogenic as tristearin.[18] Under the present conditions, the only interpretation that can be offered for the surprisingly low thrombogenicity of olive oil and mostly bovine tallow is that oleic acid, whether in its free or in its triglyceride form, is not thrombogenic. Another surprising result is the thrombogenicity of arachis oil despite its high content in oleic and linoleic acid. This, however, confirms exactly what we had previously reported[11] and it is possible that, in this fat, the small quantity of long-chain fatty acids could be very active under the present conditions.

The most deceptive result is perhaps the fact that in experiment 2 a close relationship is obvious between various parameters and the severity of thrombosis, whereas in experiment 3, where a large number of various fats were used, none of the parameters examined could really be utilized for predicting the severity of thrombosis, a result similar to that already reported with various fatty acids.[13, 15] Of course, large thromboses cannot be initiated by endotoxins in animals with a serum cholesterol level below 200 mg and with a clotting time longer than 180 sec. On the basis of a large scale experiment, however, all that can be said is that these parameters represent no more than risk factors and by no means serve to predict closely the thrombotic tendency. From our experience with the addition of free fatty acids to the diet, these results are not surprising, because we have observed that the thrombogenicity of the different fatty acids does not necessarily parallel their effect on serum cholesterol or triglycerides. If, however, as suggested by these experiments, the thrombotic tendency is mostly due to the effect of some fatty acids on blood platelets, the parameters examined, except to some extent the clotting time, do not indicate this. Nevertheless, although the plasma clotting time as performed under our conditions is a highly constant and reliable determination, it is much more affected by the environmental conditions of the animal than the thrombotic phenomenon itself and, therefore, does not appear to constitute the ideal test.

Under the present conditions, it seems clear that neither the origin of a fat nor its degree of saturation governs its lipemic, thrombogenic or atherogenic effects. The type of fatty acids it contains is probably responsible for the above-mentioned effects; but due to the number of fatty acids involved and the various interactions observed to occur with such dietary feeding,[30] many more experiments will need to be done before we can completely predict the lipemic and thrombogenic effects of certain fats or fat mixtures. Also, with so many data to deal with, the help of a computer will probably be necessary, as other investigators have realized under similar conditions.[31] Another source of error is that, except for four samples, the composition in fatty acids of the fats used here has not been determined and could be slightly different from the reported in table 3.

As regards the mortality rate and the survival time, in almost none of the present or previous experiments did we find it to be related to the severity of the occlusive thrombosis. At least on a group basis, the survival time can be correlated with the degree of lipemia, as previously observed,[10] and the mortality is probably attributable to shock. In rabbits treated by injection with bacterial endotoxin, it was also reported that the mortality rate was related to the triglyceride level.[31]

From a practical point of view, it seems essential to evaluate to what extent the present experimental model in the rat yields results that are comparable to those obtained in man and also the degree to which it could help to clarify the pathogenesis of large thrombosis and coronary disease in man. Under our conditions, after cacao butter, which has also been found to be highly atherogenic in the rabbit,[26, 32] the most thrombogenic fat is butter and, therefore, probably the other dairy products also. Dairy products represent a large portion of the dietary fat of the highly civilized countries with a high rate of coronary disease. Coconut oil, although able to induce a degree of cholesterolemia similar to that of butter, does not seem to be highly thrombogenic in man[28] or under our experimental conditions. Olive oil, like oleic acid a nonthrombogenic fat, is largely used in the diet of countries such as Italy or Greece where the incidence of coronary disease is not as high as in northern Europe and North America.

These are only a few examples of the relationships that should be underlined and, it is hoped, that will stimulate fruitful collaboration between the experimental approach on the animal and epidemiologic studies in human.

SUMMARY

Among the more common saturated fatty acids, palmitic acid appears to be the most hyperlipemic when added to the basic high-fat diet of rats. Under these conditions, stearic and especially lauric acid seem to have much less effect on both the cholesterol and triglyceride serum levels. However, the rats fed the long-chain fatty acids, palmitic and mostly stearic acids, presented the most severe phlebothromboses, initiated in these rats by the intravenous injection of a *S. typhosa* endotoxin and resulting in multiple red hepatic infarcts.

Fats richer in stearic acid, such as cacao butter and butter, are also much more thrombogenic than lard and corn oil, which contain less stearic acid and much more linoleic acid, probably an antithrombogenic fatty acid.

Among a large number of fats fed to rats under comparable conditions, the results obtained were not as clear because butter was the most thrombogenic and olive oil and bovine tallow, despite a high content in stearic acid, the least thrombogenic. The low thrombogenicity of these latter fats, despite a marked hyperlipemic effect, is probably due to their high oleic acid content. However, the thrombogenicity of a fat is probably the result of multiple fatty acid interactions and should be further analyzed.

Only with a limited number of fats could the lipemia or the plasma

clotting time be utilized to predict the thrombotic tendency of the animals. But in large-scale experiments, serum cholesterol and triglycerides, as well as the plasma clotting time, could be considered merely as risk factors. In contrast to this, there was a highly significant correlation between the survival time of the endotoxin-treated animals and their serum triglyceride level.

The effects of a few selected fats on the production of aortic fatty streaks in the rat seem to be related to the thrombogenic effect and also to the composition in fatty acids. Under the conditions of this study, neither the degree of saturation of a fat nor its origin (animal, vegetable) appears to be of any use for predicting its lipemic, thrombogenic or atherogenic effects.

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VI. Lipids — Lipides — Lipide

Dietary Saturated and Unsaturated Fatty Acids in the Production of Thrombosis and Atherosclerosis in Rat

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Introduction

Although it appears to be well documented that unsaturated dietary fats effectively decrease serum cholesterol in man, the beneficial influence of these fats on atherosclerosis in man does not seem to be clearly established as yet [3]. In animals, several studies have shown that the cholesterol level, as well as the degree of atherosclerotic lesions [1, 4, 8], depends on the degree of saturation of the dietary fat. Nevertheless, it has been reported [7, 10] that the severity of atherosclerotic lesions in rabbits does not vary with the iodine value of the fat. The difficulty in comparing various fats is that they differ, not only in their degree of saturation, but also in the chain length of the fatty acids they contain.

We showed recently [12] that with regard to lipemic and thrombotic changes in the rat, dietary addition of free fatty acids are conducive to results comparable to those obtained by the addition of esters or fats containing these same fatty acids. We also concluded that it was not necessarily the degree of saturation that was related to the incidence of thrombosis, but rather the content of a fat in palmitic and stearic acids, the most thrombogenic fatty acids. These results are in agreement with the in vitro effect of saturated fatty acids on coagulation [2], and on the production of thrombotic phenomena by the intravenous injection of these fatty acids into animals [5].

Our aim in the present experiments was to extend our results to atherosclerosis and to unsaturated fatty acids by determining in rats:

whether the addition of unsaturated fatty acids to the hyperlipemic diet could also affect the coagulation and the thrombotic tendency of the animals;

whether atherosclerosis, like the thrombotic lesions, could be influenced by the type of the dietary fatty acid used;

whether a lipemic parameter or a coagulation test could closely predict the thrombotic or atherosclerotic tendency of the animals.

Material and method:

Holtzman male rats with an initial body weight of 150 — 170 g were utilized for this study. Each table reports the pooled data of 3 different experiments. As several rats were eliminated in the course of the experiments for various reasons, the exact number of animals actually utilized for this study is listed in the corresponding table.

The rats, housed in air-conditioned quarters, were given, ad libitum, tap water and the hyperlipemic diets. The basic diet was composed of: butter 38, casein 11, cellulose[1]) 15, cholesterol 5, salt mixture[1]) 4, sodium cholate 2, sucrose 23, vitamin mixture[1]) 2 (weight %) as reported in detail elsewhere [11, 12].

The fatty acid shown in the table for each experimental group was added, in the amount of 3 %, to this basic diet at the expense of the butter.

At the end of the feeding period indicated in the corresponding table and after 17 hours' fasting, blood was removed in siliconized syringes from the jugular vein of all the rats, by a clean venipuncture, under ether anesthesia. Part of the blood, collected in a syringe containing sodium citrate, was kept for determination of the plasma clotting time as previously reported [12]. The remainder was utilized for determination of total cholesterol, triglycerides and lipoproteins (paper electrophoresis post-stained with Fat Red 7 B) as described in detail elsewhere [12].

For the induction of thrombosis after 6 weeks of diet feeding (table I), immediately after withdrawal of blood for laboratory tests the rats were given, by the same needle, 1 ml per 100 g body weight of physiologic saline containing 0.06 mg of a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide[2]). Following this endotoxin injection, some or most of the animals, depending on the group, died in 3 to 18 hours. The survivors were killed 24 hours after the injections, autopsy being performed on every animal and the red hepatic infarcts evaluated macroscopically. In the experiment for atherosclerosis, which lasted for 15 weeks (table II), the animals were killed after blood removal and were autopsied. The aorta, from the iliac arteries to the aortic valves, was opened longitudinally and the lesions graded in terms of an arbitary scale of 0 to 3, under stereoscopic microscope examination. The macroscopic readings were verified by histologic examination of the left hepatic lobe for thrombosis, and of the aorta and the heart for atherosclerosis.

Results

Effect on thrombosis (table I)

In group 7, fed stearic acid, the injection of the endotoxin induced a 56 % mortality-rate and the thrombotic lesions were very severe in this group. As in previous experiments [11, 12], the thrombi occurred at bifurcations of the large hepatic veins and were responsible for the large red hepatic infarcts observed in several lobes. In the other groups, the only significant lesions were noted with palmitic (group 6) and caprylic (group 5) acids. Independently of the severity of the lesions, the highest mortality-rate (83 %) was recorded in groups 4 and 6, which also presented the highest triglyceride levels in the serum. In groups 2 and 3 fed linoleic and linolenic acids respectively, no thrombosis occurred and the triglyceride and cholesterol levels were the lowest of all the groups. Nevertheless, no thrombosis was recorded in group 8 fed behenic acid either, although the triglyceride and cholesterol levels were the same as those of group 7 fed stearic acid and presenting the most severe lesions. As regards the lipoproteins, however, fractions $\alpha_2 + \beta$ were the most elevated in groups 5, 6 and 7, which also presented the most severe thrombotic lesions.

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[1]) The cellulose (Alphacel), the salt mixture (Wesson) and the vitamin mixture (vitamin diet fortification mixture) were all purchased from Nutritional Biochemicals Co., Cleveland, Ohio U.S.A.

[2]) Difco laboratories, Detroit, Mich., U.S.A.

Table I. Influence of dietary fatty acids on lipemia and the production of thrombosis in rat. Duration = 6 weeks

Fatty acid (Carbon atoms)	Oleic 18 (-2H)	Linoleic 18 (-4H)	Linolenic 18 (-6H)	Erucic 22 (-2H)	Caprylic 8	Palmitic 16	Stearic 18	Behenic 22
Group	1	2	3	4	5	6	7	8
Number of animals	18	18	18	6	16	18	18	12
Cholesterol (mg %)	687 ± 134	402 ± 110	379 ± 101	—	822 ± 235	1368 ± 186	717 ± 224	728 ± 67
Triglycerides (mg %)	83 ± 19	40 ± 12	61 ± 16	355 ± 72	149 ± 30	493 ± 56	125 ± 18	181 ± 13
Lipoproteins (%) $\alpha+\alpha_2$	31 ± 1.0	33 ± 1.5	30 ± 0.9	—	27 ± 1.5	20 ± 3.2	32 ± 2.0	37 ± 2.0
Lipoproteins (%) $\alpha_2+\beta$	52 ± 1.5	51 ± 1.8	52 ± 1.2	—	59 ± 2.0	69 ± 2.6	56 ± 1.6	51 ± 1.2
Plasma clotting time (sec)	173 ± 13	179 ± 15	171 ± 11	156 ± 8	183 ± 8	153 ± 15	165 ± 11	225 ± 7
Thrombosis. Severity (0—3)	0.2 ± 0.1	0	0	0.2 ± 0.1	1.2 ± 0.4	1.6 ± 0.3	2.0 ± 0.3	0
Mortality (%)	17	17	17	83	55	83	56	17

Results = Mean ± S.E.

Diet composition in addition to the fatty acid (3 %) listed in the table: butter 35, casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight %).

Table II. Influence of dietary fatty acids on lipemia and the production of aortic atherosclerosis in rat. Duration = 15 weeks

Fatty acid (Carbon atoms)	None	Oleic 18 (-2H)	Linoleic 18 (-4H)	Linolenic 18 (-6H)	Erucic 22 (-2H)	Caprylic 8	Palmitic 16	Stearic 18	Behenic 22
Group	1	2	3	4	5	6	7	8	9
Number of animals	20	17	20	22	10	22	11	10	19
Triglycerides (mg %)	152 ± 16	203 ± 28	108 ± 8	117 ± 20	308 ± 36	198 ± 29	332 ± 42	296 ± 85	90 ± 10
Lipoproteins[1] (% $\alpha + \alpha_2$)	23.4 ± 2.0	23.2 ± 1.4	25.0 ± 0.9	26.0 ± 0.8	26.2 ± 1.4	23.4 ± 1.2	—	19.2 ± 0.2	27.2 ± 0.3
Plasma clotting time (sec)	132 ± 4	165 ± 8	169 ± 10	170 ± 9	160 ± 14	130 ± 10	103 ± 9	110 ± 2	161 ± 15
Body weight gain (g)	86 ± 6	74 ± 6	78 ± 6	63 ± 5	97 ± 9	67 ± 5	72 ± 9	97 ± 10	119 ± 9
Atherosclerosis. Severity (0—3)	1.6 ± 0.2	1.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	2.0 ± 0.3	1.8 ± 0.3	0.9 ± 0.2

[1] Only 6 rats per group

Results = Mean ± S.E.

Diet composition in addition to the fatty acid (3 %) listed in the table, included at the expense of butter (butter 38, casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight %).

Effect on atherosclerosis (table II)

The atherosclerotic lesions observed here consisted of fatty streaks, composed mostly of one or several layers of foam cells covered by endothelial cells, with little fibrosis. These lesions are easily detectable by stereoscopic microscope examination and, in the most severe cases, are present all along the aorta. In the group given behenic acid, however, although the aortic lesions were very mild, unusual atheromatous plaques could be observed near the aortic valves and particularly in the Valsalva's sinus.

The most severe aortic lesions were recorded in groups 7 and 8, fed palmitic and stearic acids, respectively. In group 8, the percentage of $\alpha + \alpha_1$ lipoproteins was the lowest of all the groups and the plasma clotting time was also significantly lower in the rats of groups 7 and 8 than in any other group. The least severe lesions were recorded in groups 3 and 4, fed linoleic and linolenic acids respectively. The triglyceride level was also the lowest in these two groups and the plasma clotting time the highest of all the groups. Their percentage of $\alpha + \alpha_1$ lipoproteins was among the most elevated, but was higher in group 9 fed behenic acid.

Among the animals fed the saturated fatty acids (group 6 to 9), the smallest gain in body weight was recorded in group 6, fed caprylic acid, and the highest was seen in group 9, fed behenic acid. Among the unsaturated fatty acids, the longest chain fatty acid (erucic) also induced the highest weight gain.

Discussion

In the present experiment, in the rat, the dietary addition of fatty acids at 3 %, instead of at 8 % as in the previous study [12], and using butter instead of lard as the basic fat, was conducive to the same result obtained in that study, namely, that among the saturated fatty acids utilized, only palmitic and stearic acids appear to be highly thrombogenic. However, under the present conditions the lesions in the group fed caprylic acid were more severe than those previously observed [8]. One additional result obtained here is that palmitic and stearic acids were also the most atherogenic fatty acids, despite the fact that the hyperlipemia they induced was no more severe than that obtained with erucic acid. The marked atherogenicity of stearic acid in the rabbit has already been reported [7].

In all the groups fed the unsaturated fatty acids, thrombotic lesions were either absent or of very mild degree. In these groups, the degree of atherosclerosis was also less severe than that observed in the control group fed butter alone. However, the most marked preventive effect was obtained by feeding linoleic and linolenic acids, a finding that serves to confirm and explain the results of several workers [1, 4, 8, 10, 9] concerning fats of different origins rather than purified fatty acids. These results are also in agreement with those reported recently in man [6], which indicated that oleic and erucic acids do not decrease the serum cholesterol level. In the present experiments, at 6 weeks, the serum cholesterol of the animals fed oleic acid was not significantly different from that of animals fed stearic acid.

Summary

Inclusion of free fatty acids in the hyperlipemic diet of rats was able to markedly change, not only the lipemic, but also the thrombotic and atherosclerotic response of these animals.

There was a close relationship between the thrombogenicity and the atherogenicity of a fatty acid. Among the fatty acids examined, linoleic and linolenic acids were the least [illegible] and also the least atherogenic, while palmitic and stearic acids were the most [illegible] in [illegible].

In the present experiments, although lipemia was lowest in the groups with the least severe lesions, neither the lipemic parameters examined nor the clotting time, when regarded as separate entities, were able to closely predict the thrombotic or atherosclerotic tendency of the animals. However, when these parameters were considered all together, they gave a good indication of the severity of the lesions to be expected.

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METABOLIZABLE ENERGY VALUES OF FATS AND FATTY ACIDS FOR CHICKENS

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Studies on the metabolizable energy content of various fats and fatty acids have been conducted during the past year as part of a general program in this laboratory to determine the energy values of purified nutrients and practical feedstuffs for the chicken.

Metabolizable energy was determined by bomb calorimetry using the chromic oxide indicator method and correcting for nitrogen retention.[1] Metabolizable energy values for various fats and fatty acids were obtained by feeding chicks semi-purified diets in which the given fat was substituted either weight for weight or isocalorically for glucose (Cerelose). From the difference in metabolizable energy content of the reference diet and fat-containing diets, metabolizable energy values for fats and fatty acids were determined, assuming the metabolizable energy value of glucose, the standard reference substance, to be 3.64 Cal. per gram on a dry matter basis.[2]

By comparing the metabolism energy value of fat to its gross energy, a measure of fat utilization is obtained. Such values reflect not only the utilization of fat itself, but also the effect of fat on the digestibility of other components of the diet. More usually fat utilization has been studied by digestibility, from which a digestibility coefficient is determined by comparing the amount of fat excreted to the amount ingested after correcting for metabolic fat. In the present work, simultaneous determinations of fat utilization by the two methods were used. They were generally in good agreement, but showed that utilization expressed as metabolizable energy was slightly less than the digestibility of the fat. Three possible explanations can be postulated for the difference between the two methods. Either, (a) the high level of fat adversely affected the utilization of some other nutrient in the diet, or (b) the metabolizable energy value used for the reference substance (glucose) may be slightly low. On the other hand, (c) digestibility coefficients could be erroneously high if determination of fecal fat is incomplete.

Summarized in Table 1 are the metabolizable energy

values of the various fats and fatty acids studied together with their utilization as indicated by metabolizable energy as a percentage of gross energy.

All values listed in Table 1 were obtained with fat or fatty acid included in the diet at a level of approximately 20%. Anderson[3] found no difference in the metabolizable energy obtained for stabilized tallow when fed at levels of 10 and 20% of the diet. He reported values of 3260 and 3240 Cal/lb. respectively. In the case of other feedstuffs as well, level of substitution has not affected determination of the energy value, except for the readily predictable relationship that precision increases with higher levels of substitution.

The metabolizable energy values of the various fats and fatty acids represent values for the chick at 3-4 weeks of age except in the case of feed grade tallow, lard and corn oil where a systematic study was made to determine their utilization at 2, 4, 6 and 8 weeks of age.[4] Results of this study showed that only the utilization of tallow was affected by the age of the chick, older chicks being better able to utilize tallow than younger chicks. Lard and corn oil were equally well utilized by all age groups.

Of the edible fats studied, soybean oil had the highest energy value for the chick while corn oil and lard were also very well utilized. Hydrogenated vegetable fat, although more unsaturated than lard, was less well utilized.

Among the inedible fats, the fish oils had the highest energy value, however, they were less well utilized than either soybean oil, corn oil or lard. On the average, fish oils were utilized to 86% which is in marked contrast to the human, rat and guinea pig which utilize cod liver oil to 98%, 98% and 94% respectively.[5] The two samples of tallow had the lowest energy value in this group of fats, ranging from 2860 to 3000 Calories per pound. Pure beef tallow had a lower energy value than feed grade tallow for the four week old chick, which was to be expected since the latter contains some pork fat, which is better utilized by the chick than beef tallow. Hydrolyzed fat (a feed grade fat composed of approximately equal parts of fatty acids and neutral fat from animal and vegetable sources), acidulated soybean soapstock and soybean lecithin had slightly higher energy values than the tallows.

In an effort to gain information on fat digestion and absorption in the chick, fatty acid mixtures derived from beef

tallow, lard and soybean oil were compared with the respective intact fats. The energy values of the mixtures of fatty acids were much lower than the energy values of the respective neutral fats. From the composition data for these mixtures it appears that the free saturated fatty acids were not utilized even in the presence of unsaturated fatty acids. This is consistent with the finding that stearic and palmitic acids when fed singly are not utilized by the chick at 4 weeks of age while oleic acid is utilized to about 88%.[6] Sunde[7] has also reported that stearic acid is poorly utilized by the chick.

The metabolizable energy value obtained for a fat depends on two physiological processes, digestion and absorption. Since Mattson[8] discovered that pancreatic lipase is specific for reactions occurring at the primary hydroxyl groups of triglycerides, a relatively clear picture of the mechanism of fat digestion in the rat is available; however, the process of fat absorption is still obscure. According to the lipolytic theory, fat is completely hydrolyzed in the intestinal tract to free fatty acids and glycerol. According to the partition theory, digestion proceeds only far enough to yield sufficient monoglycerides and free fatty acids which together with the bile salts emulsify the remaining triglycerides. According to this theory, the main bulk of fat is absorbed as the triglyceride. Recently Reiser[9] has concluded from experiments using radioactive techniques that only 3% of tripalmitin was absorbed unhydrolyzed by the rat. He postulated that fat is broken down to free fatty acids and monoglycerides which are resynthesized in the intestinal mucosa to form neutral fat.

The poor utilization of free fatty acids which was obtained in these studies supports the theory that either neutral fat or some intermediate breakdown product of triglycerides is required for absorption of free fatty acids. Determination of the component or components of neutral fat which are required awaits further study.

Up to recent years, two theories have been used to attempt to explain differences in digestibility of intact fats. Crockett and Deuel[10] and Langworthy[11] postulated that digestibility is inversely proportional to and dependent on melting point, while Hoagland and Snider[12] and Mattil[13] postulated that digestibility is limited by the amount of saturated fatty acids of carbon chain 18 or longer. Application of either of these theories to the fats studied does not explain the similar utili-

zation of lard and corn oil by the chick, the large difference in utilization of lard and tallow, or the differences in utilization of soybean oil, corn oil and fish oils. Recently, however, Calloway and coworkers[14] have concluded that digestibility is primarily dependent on the amount and chain length of saturated fatty acids and their arrangement within the glyceride structure. Thus, further studies may show that the difference in digestibility of lard and tallow which cannot be explained entirely by their saturated fatty acid content or melting point is related to the arrangement of fatty acids in the glyceride molecule. Similarly, such studies may reveal why lard containing 35% saturated fatty acids is as well utilized as corn oil containing 11% saturated fatty acids.

SUMMARY

Metabolizable energy values of several well characterized fats, mixtures of fatty acids and individual samples of oleic, palmitic and stearic acid have been determined for the chick. Soybean oil, corn oil and lard were of high energy value for the chick. A sample of hydrogenated vegetable fat, although more unsaturated than lard, was less well utilized. Of the inedible fats or fat by-products studied, fish oils had the highest energy value, hydrolyzed fat, acidulated soybean soapstock and soybean lecithin were intermediate, while beef tallow had the lowest energy value. Results indicate that some factor in addition to melting point and saturated fatty acid content determines the relative metabolizable energy values of various fats.

Mixtures of fatty acids had much lower energy values than the neutral fats from which they were derived, supporting the theory that only partial hydrolysis of fat occurs in the digestive process. Composition data indicate that saturated fatty acids in such mixtures are not utilized. This is consistent with the finding that stearic and palmitic acids when fed singly are not utilized by the chick.

The utilization of fats and fatty acids measured by metabolizable energy value agreed closely with digestibility coefficients determined simultaneously. This shows that the inclusion of fat in the diet had no major effect on utilization of other components.

TABLE 1. Metabolizable energy values and utilization of fats and fatty acids

	Iodine number	M.E. Cal./lb.	Utilization %
Edible fats			
Corn oil	122	3950	92.9
Degummed soybean oil	133	4210	98.8
Lard	65	3980	93.4
Hydrogenated fat	69	3250	76.2
Inedible fats			
Feed grade tallow	52	2990**	70.3
		3230***	76.2
Beef tallow	44	2860	67.0
Hydrolyzed fat	61	3230	77.8
Menhaden oil	169	3700	87.8
Red fish oil	120	3580	83.4
Cod liver oil	154	3690	87.0
Fatty acids			
Palmitic		−9	−0.2
Stearic		−17	−3.9
Oleic		3770	88.4
Soybean F.A.	131	3750	89.3
Lard F.A.*	67	2730	64.8
Tallow F.A.*	44	2010	47.7
Miscellaneous			
Acidulated soybean soapstock	124	3150	75.3
Soybean lecithin		3060	86.5

* Derived by hydrolysis from the samples of beef tallow and lard studied.
** Young chicks, 2-4 weeks old.
*** Older chicks, 6-8 weeks old, and hens.

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The Utilization of Corn Oil, Lard and Tallow by Chickens of Various Ages

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QUANTITATIVE data on the utilization of fats and fatty acids by poultry are quite limited. Whitson *et al.* (1943) described an index method for determination of digestibility using barium sulfate, and showed that soybean oil is highly digested and absorbed by growing chickens. Duckworth *et al.* (1950) found that linseed oil is well utilized by chicks, but mutton tallow is much less well absorbed. Their data indicated that the absorbability of mutton fat increased with age. Carver *et al.* (1955) found that beef tallow was well utilized when added at a level of 3% to a practical broiler ration. Hydrogenated tallow and the fatty acids of hydrogenated tallow were less well absorbed, while oleic acid showed high absorbability. Sunde (1956) obtained evidence that the hard fats, tallow and hydrogenated tallow, when fed at a level of 5% in a practical chick ration were less well utilized than softer inedible fats classified as greases. Stearic acid was poorly absorbed in his work, while oleic, linoleic, and linolenic acids were highly absorbed. March and Biely (1957) showed large differences in apparent absorbability between corn oil (85–92%), beef tallow (73–75%) and hydrogenated fat (50%) when fed to growing chicks.

The studies to be described were conducted to determine the utilization of corn

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oil, lard and tallow by chickens of different ages. Two methods for studying fat utilization were employed: (1) metabolizable energy value determined by bomb calorimetry, and (2) determination of absorbability by analysis of diets and excreta for fats and fatty acids. The purpose of using both methods to estimate fat utilization was to compare their usefulness, and to determine whether the fats affected the utilization of other dietary constituents.

MATERIALS AND METHODS

One experiment was conducted with male crossbred (RIR × BPR) chicks. They were randomly distributed into lots of 10 chicks each and housed in electrically heated, thermostatically controlled battery brooders with raised wire screen floors in a temperature controlled laboratory. Two duplicate groups were fed each of the experimental diets, and feed and water were supplied *ad libitum*. Another experiment was conducted with adult laying hens. Single Comb White Leghorns, 9 months old, were used for this purpose. Two duplicate groups of two hens each, maintained in wire-floored layer cages in a heated laboratory room, were used for each dietary treatment.

The composition of the experimental diets is shown in Table 1. The low fat reference diet was the same as diet E9 described in detail by Hill *et al.* (1960). Preliminary work had indicated that tallow had a metabolizable energy for chicks of approximately 7.7 Calories per gram, so the fats were incorporated in the respective diets on the assumption that they all had approximately this value. On this basis, 17.5 parts of each of the respective fats replaced 36.9 parts of glucose on a dry matter basis to achieve approximately equicaloric substitution of fat for carbohydrate, using the value 3.64 Calories per gram of glucose previously determined in this laboratory (Anderson, Hill and Renner, 1958). Corn oil (Mazola) and lard were obtained through food distribution channels. The tallow was a feed grade material composed of approximately 80% beef tallow and 20% inedible pork fat.

TABLE 1.—*Composition of experimental diets*

	Parts in	
	Low-fat reference diet	Fat-supplemented diets
Constant components[1]	56.6	56.6
Glucose (Cerelose)	43.4	6.5
Fat (corn oil, lard, or tallow)	—	17.5
Total parts	100.0	80.6

[1] Ground wheat 9, dehulled soybean oil meal 15.8, crude casein 10.5, gelatin 2.5, fish meal (menhaden) 4, fish solubles 2, dried brewers yeast 2.5, dried whole whey 2, hydrogenated vegetable shortening 2.5, BHT .01, DPPD .01, mineral supplements 4, vitamin mixture .8, chromic oxide "bread" 1. Composition of mineral and vitamin supplements and chromic oxide "bread" described in detail by Hill, Anderson, Renner and Carew (1960).

During the course of the experiment with growing chicks, excreta were collected during the first four days of the second, fourth, sixth and eighth week of life. In the experiment with hens, excreta collections were made after they had been fed the experimental diets for 1 week. Chromic oxide was incorporated in each of the diets at a level of approximately 0.3% as an index substance in order to establish the amount of excreta produced per unit of feed intake without need for quantitative collection of the excreta or quantitative measurement of feed intake. The methods of processing excreta, conducting chemical analyses for moisture, nitrogen, combustible energy, and chromic oxide, and the method of computing metabolizable energy of the diet from these data, have been described previously in detail (Hill and Anderson, 1958; Hill, Anderson, Renner and Carew, 1960). The metabolizable energy of each of the three fats was computed as

follows: the metabolizable energy per gram of the low fat reference diet minus the metabolizable energy of the glucose replaced by fat (3.64 × 0.369) gave the calculated value of the constant portion of the diet (0.631 grams); the metabolizable energy per 0.806 grams of fat-supplemented diet minus the metabolizable energy of 0.631 grams of constant portion gave the metabolizable energy of 0.175 grams of the respective fat, from which the value per gram was derived. The utilization of each fat was computed from its metabolizable energy value as a percentage of its gross combustible energy using the determined values 9.44, 9.42 and 9.34 Calories per gram for corn oil, lard and tallow, respectively.

Absorbability of the various fats was determined by appropriate analysis of diets and excreta for fats and fatty acids. Dietary fat was determined by ether extraction in a Goldfisch extraction apparatus. The total fat excreted, including neutral fat and fatty acids, was determined by the following modification of the method of Fowweather and Anderson (1946):

> A two gram sample of dried feces ground to pass a 40 mesh screen was mixed with 3 cc. water and 1 cc. concentrated HCl in a small mortar and left for 30 min. Six grams anhydrous $MgSO_4$ was then added and the ground mixture allowed to stand overnight in an oven at 40°C. The mixture was then extracted for 3 hrs. with petroleum ether (60°–70°C.) in a Goldfisch apparatus after which excess solvent was removed in a steam bath and the extracts dried for 30 minutes in a forced air oven at 105°C.

Correction for endogenous fat and fecal fat contributed by constant diet ingredients was made from the data obtained for the low fat reference diet. Because the level of chromic oxide was essentially identical in all diets in respect to the non-glucose, non-fat ingredients, the fat excretion per unit of index substance on the low fat reference diet could be used to estimate endogenous and basal unabsorbed fat. This estimate was subtracted from the fat excreted per unit of chromic oxide by the groups fed the fat supplemented diets. The correction was of the order of 3–5% of the dietary fat for chicks and 2% for hens at the level of supplementation employed in this experiment. Absorbability was computed from the corrected fat excretion per unit of food intake as compared to the level of the respective fat contained in the diet.

At the completion of the experiment, the combined data on the utilization of all fats by the chick were subjected to an analysis of variance (Snedecor, 1956). The data for the hens were analyzed in the same way.

RESULTS AND DISCUSSION

Summarized in Table 2 are data showing the utilization of corn oil by growing chicks of various ages and adult hens. Analysis of variance (Snedecor, 1956) showed that the metabolizable energy value of corn oil for chicks did not change significantly in the course of the experiment from two to eight weeks of age. Expressed as a percentage of gross combustible energy, the metabolizable energy value of corn oil ranged from 91–96% for chicks and was 90% for the adult hen. Also shown in Table 2 are data on the absorbability of corn oil, based on determinations of fat and fatty acids in the diets and excreta. Percentage utilization by this technique ranged from 94–98% for chicks and was 94% for adult hens.

Similar data on the utilization of lard are shown in Table 3. Utilization as determined by metabolizable energy value ranged from 89–92% for chicks in the period from two to eight weeks of age, and no change with age was evident over this period. A lower metabolizable energy value

TABLE 2.—*Utilization of corn oil by chickens of various ages*

Age in weeks	Energy utilization: Metabolizable energy		Energy utilization: Percent of gross energy[1]		Absorbability[2]	
	Cal./gm.				%	
2 weeks	8.89		94		97	
	8.36	8.62[3]	89	91	92	94
4 weeks	8.94		95		98	
	9.15	9.04	97	96	98	98
6 weeks	8.91		94		97	
	8.50	8.70	90	92	96	97
8 weeks	8.69		92		95	
	8.72	8.70	92	92	95	95
38 weeks (adult hens)	8.56		91		95	
	8.42	8.49	89	90	94	94

[1] Metabolizable energy as a percentage of gross energy of corn oil (9.44 Cal./gm.).
[2] Computed from dietary fat and total fecal fat, correcting for fecal fat from low-fat reference diet.
[3] Underlined values are averages of duplicate lots; individual replicate values are in the respective left columns.

equal to approximately 82% utilization of gross energy was shown by the adult hen. That the lower metabolizable energy value shown by hens was due to reduced absorption of the fat is shown by the data for absorbability. While the chicks absorbed lard to the extent of 90–95%, the hens showed only 85% utilization by this measure.

The observation that the utilization of lard by the hen was less efficient than by the growing chick was quite unexpected. In subsequent work using a different source of lard, hens have shown a metabolizable energy for lard equal to that obtained with chicks. However, no ready explanation for the difference found in the present experiment is apparent.

Data on the utilization of tallow by chicks and hens are shown in Table 4. In contrast to the absence of any trend in fat utilization with age of chicks for other fats, the utilization of tallow increased with age in the range from two to eight weeks of age. Utilization as measured either by metabolizable energy or absorbability showed that the chick eight weeks of age is equal to the hen in efficiency of utilization of this type of fat.

The increase in tallow utilization as the chick grows older is somewhat similar to the data reported by Duckworth *et al.* (1950), and described above. Bose and Subrahmanyan (1950) also noted a similar improvement with age in the digestibility of peanut oil and the saturated fatty acids from hydrogenated peanut oil in the rat. Norcia and Lundberg (1954) found that the utilization of tripalmitin by the rat was increased with time.

TABLE 3.—*Utilization of lard by chickens of various ages*

Age in weeks	Energy utilization: Metabolizable energy		Energy utilization: Percent of gross energy[1]		Absorbability[2]	
	Cal./gm.				%	
2 weeks	8.69		92		91	
	8.55	8.62[3]	91	92	90	90
4 weeks	8.30		88		91	
	8.50	8.40	90	89	92	91
6 weeks	8.46		90		93	
	8.81	8.63	94	92	96	95
8 weeks	8.67		92		92	
	8.72	8.70	93	92	92	92
38 weeks (adult hens)	7.81		83		86	
	7.72	7.76	82	82	85	85

[1] Metabolizable energy as a percentage of gross energy of lard (9.42 Cal./gm.).
[2] Computed from dietary fat and total fecal fat, correcting for fecal fat from low-fat reference diet.
[3] Underlined values are averages of duplicate lots; individual replicate values are in the respective left columns.

TABLE 4.—*Utilization of tallow by chickens of various ages*

Age in weeks	Energy utilization: Metabolizable energy		Energy utilization: Percent of gross energy[1]		Absorbability[2]	
	Cal./gm.				%	
2 weeks	6.18 6.97	6.58[3]	66 75	70	65 74	70
4 weeks	6.45 6.69	6.57	69 72	70	74 74	74
6 weeks	7.15 6.69	6.92	77 72	74	80 70	75
8 weeks	7.46 7.18	7.32	80 77	78	83 80	82
38 weeks (adult hens)	7.15 7.23	7.19	77 77	77	80 82	81

[1] Metabolizable energy as a percentage of gross energy of tallow (9.34 Cal./gm.).
[2] Computed from dietary fat and total fecal fat, correcting for fecal fat from low-fat reference diet.
[3] Underlined values are averages of duplicate lots; individual replicate values are in the respective left columns.

Comparison of the two methods for studying fat utilization showed that metabolizable energy and absorbability were quite comparable for chicks fed lard or tallow. However, statistical analysis showed that values for the utilization of corn oil by chicks and of all three fats by hens were slightly but significantly higher when determined by absorbability.

Three possible explanations to account for differences between the two methods can be advanced. It is possible that the value 3.64 Calories per gram glucose dry matter used as the reference standard may be slightly lower than its true value. In this case, any adjustment in the value of the reference standard would result in a corresponding change in the value of substances measured against it. Secondly, the high level of fat employed might have had an adverse effect on the utilization of some other component of the diet; this would have the effect of reducing the apparent metabolizable energy value of fat while the absorbability measurement would be unaffected. It is also possible that the method used to determine fat in the excreta failed to extract all of the fecal lipids. In this case the method would underestimate the unabsorbed fats and therefore overestimate the true absorption.

In the case of the hen it is not possible to choose among these three explanations. In the chick, however, since the values obtained for utilization of lard and tallow by the two methods were the same, it is unlikely that the energy value of glucose is contributing to the difference between methods in the utilization of corn oil. However, the discrepancy was relatively small for both chicks and hens, and it is evident from the data presented that metabolizable energy value and absorbability of the respective fats are closely related.

Any effect of high dietary fat levels on the digestion and absorption of other nutrients by the chicken is evidently small so far as over-all energy utilization is concerned. If the discrepancy discussed above is due to such an effect, its direction is negative since the metabolizable energy value of fat gave an index of utilization which was slightly lower than its apparent absorbability.

SUMMARY

The utilization of corn oil, lard and tallow by growing chicks of various ages and by adult hens has been studied by determining the metabolizable energy value of the fats and their absorbabilities.

Age affected only the utilization of tallow by the growing chick. In early life, utilization of this fat was lower than in the adult. By the time the chick was eight weeks of age it was able to utilize tallow

equally as well as the adult hen.

Metabolizable energy and absorbability were comparable for chicks fed lard and tallow. Values for the utilization of corn oil by chicks and for corn oil, lard and tallow by hens were slightly higher when determined by absorbability. From the size and nature of the difference, it appears that a high dietary level of fat does not have any major effect on the digestion and absorption of other nutrients, and whatever slight effect may occur is negative.

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Comparative Value of Dietary Rapeseed Oil, Sunflower Seed Oil, Soybean Oil and Animal Tallow for Chickens

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Stimulation of chick growth by supplementing rations with fat sources such as animal tallow, corn oil and soybean oil has been demonstrated (Pepper et al., '53; Aitken et al., '54; Rand et al., '58; Carew et al., '59). Little information is available concerning the influence of dietary rapeseed oil or sunflower seed oil on chick growth. Correspondingly, there is lack of information with respect to the influence these two fat sources may exert on metabolizable energy content of chick rations and on the composition of carcass adipose tissue.

The results reported herein compare the effects of dietary animal tallow, soybean oil, rapeseed oil and sunflower seed oil on chick performance, metabolizable energy content of the ration and fatty acid composition of chick carcass adipose tissue.

EXPERIMENTAL

Male broiler chicks obtained from a commercial hatchery were used in this study. At one day of age, 15 cockerels were randomly assigned to each experimental pen, wing-banded, group-weighed and placed in electrically heated brooder batteries with raised, wire-mesh floors. The cockerels remained in these pens until 4 weeks of age, at which time they were transferred to broiler finishing batteries. Each of the 9 ration treatments were assigned to the experimental pens according to a randomized block design. Weight gain and feed consumption data were recorded bi-weekly. Feed and water were supplied ad libitum.

The basal ration contained 3.1% of fat and 22.2% of protein. This ration contained the following ingredients expressed as percentage of the total ration: ground oat groats, 36.5; ground wheat, 38.0; soybean meal (45% protein), 13.0; meat meal (50% protein), 5.0; fish meal (70% protein), 2.5; dehydrated alfalfa meal (17% protein), 2.0; dicalcium phosphate, 1.5; vitamin premix, 1.0; salt premix, 0.5. The vitamin premix contributed the following per kilogram of ration: vitamin A, 7150 IU; vitamin D_3, 818 ICU; vitamin E, 22 IU; and the following expressed as milligrams per kilogram of ration; riboflavin, 5.5; niacin, 16.5; pantothenic acid, 11; choline, 275; menadione, 1.1; vitamin B_{12}, 0.01. The salt premix supplied the following, expressed as milligrams per kilogram of ration: sodium chloride, 4,600; manganese, 120; iron, 17; zinc, 11; copper, 6.6. Each fat source was substituted in the basal ration for an equivalent weight of wheat, and levels of wheat and soybean meal were adjusted to maintain a constant percentage of protein in the ration. Stabilized animal tallow, degummed soybean oil, degummed rapeseed oil and crude sunflower seed oil[1] were each tested at 4 and 8% of the ration.

The procedure described by Hill and Anderson ('58) was used for determining the metabolizable energy of the rations. This entailed the use of chromic oxide as an index substance in the rations (to establish the ratio of excreta produced per unit of feed intake), determining gross energy of feed and excreta in a Par adiabatic colorimeter, and computation of metabolizable energy values with correction being made for nitrogen retention. Nitrogen in feed and excreta was determined by the macro-Kjeldalh technique.

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[1] Soybean oil, rapeseed oil and sunflower seed oil kindly supplied by Co-operative Vegetable Oils Ltd., Altona, Manitoba.

To facilitate determination of metabolizable energy, total excrement was collected from each experimental pen for 5 consecutive days during the 4th and again during the 6th week of the experiment. Metabolizable energy of the rations was determined at the end of each 5-day collection interval as described previously.

The experiment was terminated when the cockerels were 8 weeks old. At this time, three randomly selected cockerels from each experimental pen were killed and the thyroid glands excised and weighed immediately. At the same time, approximately 15 gm of abdominal adipose tissue were removed from each cockerel. The samples of adipose tissue obtained from the three cockerels per experimental pen were pooled to form one composite sample. Fat was extracted from the composite samples according to the method described by the Association of Official Agricultural Chemists ('60). Quantitative analysis for fatty acid composition of the fat sources used in this investigation and adipose tissue was accomplished by gas chromatography. A Beckman GC-2 gas chromatograph was used and fatty acids (as methyl esters) were separated on a butanediol succinate polyester column with a flow rate of 190 ml per minute and a temperature of 200°C.

Data were subjected to analysis of variance (Snedecor, '56) and multiple comparisons made according to Duncan ('55).

RESULTS AND DISCUSSION

The data presented in table 1 show that weight gain and efficiency of feed utilization of chicks from zero to 8 weeks of age were improved by including various fat sources in the ration. In the case of weight gain, only the addition of 4% of animal tallow failed to result in a significant ($P < 0.05$) increase over the basal group. Efficiency of feed utilization was significantly ($P < 0.05$) improved by all fat additions except when 4% of animal tallow or 4% of rapeseed oil were used. The marked improvement in weight gain and feed efficiency due to adding soybean oil to the ration agrees with observations of Dam et al. ('59) and Carew et al. ('59). Aitken et al. ('54) reported a substantial stimulation of growth and increased efficiency of feed utilization of broilers when beef tallow was used at 10% of the ration. The present study indicates that 8% of dietary animal tallow stimulated growth and improved feed efficiency but that a level of 4% was relatively ineffective.

When used at 4% of the ration, rapeseed oil and sunflower seed oil were as effective in promoting weight gains as 4% of soybean oil and were more effective than 4% of animal tallow. All fat sources were similar in their growth stimulating properties when included in the ration at the 8% level. Rapeseed oil, which contains a large proportion of erucic (13-docosenoic) acid in its total fatty acid complement, was reported by Deuel et al. ('48), Thomasson ('55) and Beare et al. ('59b) to decrease body weight gain when fed to rats. Beare et al. ('59a) presented data showing that relatively high levels (5 to 11% of total ration) of ethyl erucate markedly retarded

TABLE 1

Comparative effects of dietary fats on weight gain, efficiency of feed utilization and thyroid gland weight of cockerels

Ration treatment	Av. weight gain 0–8 weeks	Feed/gain 0–8 weeks	Av. thyroid gland weight
	gm		*mg/kg body weight*
Basal (no added fat)	1396 ± 38[1]	2.14 ± 0.04	7.9 ± 1.3
Animal tallow, 4%	1412 ± 7	2.07 ± 0.04	7.4 ± 1.7
Animal tallow, 8%	1543 ± 25[2]	2.00 ± 0.02[2]	8.3 ± 1.5
Soybean oil, 4%	1485 ± 35[2]	2.00 ± 0.01[2]	9.7 ± 2.2
Soybean oil, 8%	1525 ± 28[2]	1.97 ± 0.02[2]	8.5 ± 1.4
Rapeseed oil, 4%	1499 ± 34[2]	2.06 ± 0.03	10.2 ± 1.7
Rapeseed oil, 8%	1513 ± 39[2]	1.94 ± 0.01[2]	7.7 ± 0.7
Sunflower seed oil, 4%	1560 ± 25[2]	1.97 ± 0.04[2]	12.0 ± 1.6
Sunflower seed oil, 8%	1477 ± 35[2]	2.00 ± 0.06[2]	7.1 ± 1.1

[1] Figures preceded by ± sign are standard errors of the mean.
[2] Difference between this mean and the basal group significant at $P < 0.05$.

growth of young rats and concluded that growth inhibition caused by high dietary levels of rapeseed oil was due to erucic acid. These workers also noted that depression of weight gain could be explained by reduced feed consumption of animals receiving a diet containing rapeseed oil or ethyl erucate.

The rapeseed oil used in the present investigation supplied about 2.5% of erucic acid to the total ration when the oil comprised 8% of the diet. There was no apparent harmful influence exerted by this amount of dietary erucic acid; in fact, weight gains were increased slightly.

Thyroid gland weights shown in table 1 indicate that little, if any, of the goitrogenic material normally found in whole rapeseed and rapeseed oil meal was present in the rapeseed oil tested. None of the fat sources studied caused any appreciable change in thyroid gland weight.

The data on metabolizable energy of the rations are shown in table 2. Values obtained for each ration during the 4th and 6th weeks of the experiment were pooled to arrive at the given data. This procedure appeared to be justified because the metabolizable energy values for each ration as determined during the 4th and 6th week were not significantly ($P < 0.05$) different.

When added to the ration at a 4% level, rapeseed oil and animal tallow were quite similar with respect to resulting metabolizable energy values. The ration containing 4% of sunflower seed oil had a metabolizable energy value slightly higher than the ration containing 4% of animal tallow and significantly ($P < 0.05$) higher than the ration with 4% of rapeseed oil. However, very close similarity between rapeseed oil, animal tallow and sunflower seed oil was observed when the fat sources comprised 8% of the ration, indicating that rapeseed oil and sunflower seed oil, as used in this investigation, are essentially equivalent to animal tallow in terms of resulting metabolizable energy. Deuel et al. ('48, '49) reported that rapeseed oil fed to the rat had a lower digestibility coefficient and slower rate of absorption than many other fat sources tested, whereas in humans the digestibility coefficient for rapeseed oil was about 99%. Beare et al. ('57) observed increased lipid excretion by rats receiving rations containing 10 to 20% of rapeseed oil, but this increase did not occur when lower levels (less than 8%) of dietary rapeseed oil were used. The comparative growth and ration metabolizable energy data presented herein indicate that energy supplied by up to 8% of dietary rapeseed oil was as available to the chick for subsequent utilization as energy derived from comparable levels of dietary animal tallow or sunflower seed oil.

Sibbald et al. ('61) reported that crude soybean oil possessed a considerably higher metabolizable energy value for chickens than animal tallow. In the present study, rations containing 4 to 8% of soybean oil had significantly higher metabolizable energy values than when corresponding levels of animal tallow, rapeseed oil or sunflower seed oil were fed. Thus, it would appear that soybean oil has a higher metabolizable energy content per se than other fat sources tested or that soybean oil exerts an influence on other ration ingredients whereby ration metabolizable energy is increased over that realized when other fat sources are used. But, although soybean oil significantly increased ration metabolizable energy as compared with other fat sources tested, soybean oil did not consistently stimulate growth any more than did comparable levels of animal tallow, rapeseed oil and sunflower seed oil.

The fatty acid composition of the dietary fat sources and the abdominal adipose tissue are presented in table 3. In general, fatty acid composition of the adipose tissue reflected that of the dietary fat, particularly when an 8% level of the fat source was used. Cruickshank ('34), Adamson et al. ('61) and Rogler and Carrick ('61) reported that, on the basis of iodine number, degree of unsaturation of dietary fatty acids had a marked influence on carcass fat of chickens. Feigenbaum and Fisher ('59) using gas chromotography, also observed that the fatty acid composition of carcass fat was influenced by the composition of the dietary fat.

The data presented herein show that in comparison with the adipose tissue of chicks receiving the basal ration, adipose tissue of chicks receiving animal tallow reflected the relatively high stearic-oleic acid ($C_{18} + C_{18:1}$) and low linoleic acid ($C_{18:2}$)

TABLE 2

Metabolizable energy of rations containing different fat sources as determined with cockerels from 4 to 8 weeks of age

	Ration treatment[1]								
	Basal (no added fat)	4% Rapeseed oil	4% Animal tallow	4% Sunflower seed oil	4% Soybean oil	8% Rapeseed oil	8% Animal tallow	8% Sunflower seed oil	8% Soybean oil
Metabolizable energy, Cal./gm dry ration	2.89 ± 0.02[2]	2.99 ± 0.02	3.04 ± 0.03	3.11 ± 0.01	3.22 ± 0.02	3.25 ± 0.07	3.26 ± 0.01	3.29 ± 0.02	3.45 ± 0.04

[1] All means not underscored by the same line are significantly different at $P < 0.05$.
[2] Figures preceded by ± sign are standard errors of the mean.

TABLE 3

Fatty acid composition of fat sources used in rations and abdominal adipose tissue of 8-week-old chicks

	C_{14}	$C_{16}+C_{16:1}$	$C_{18}+C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:4}$	$C_{18:1}$
	Fatty acid composition of fat sources (percentage of total methyl esters)						
Fat source added to ration							
Animal tallow	3.75	33.14	61.88	1.23	—	—	—
Soybean oil	—	11.11	28.87	52.44	7.58	—	—
Rapeseed oil	4.30	26.57	20.09	6.50	5.34	3.72	31.48
Sunflower seed oil	—	6.80	20.93	70.47	1.60	—	—
	Fatty acid composition of abdominal adipose tissue (percentage of total methyl esters)						
Ration treatments							
Basal (no added fat)	1.51 ± 0.33[1]	34.79 ± 1.26	47.62 ± 0.61	16.08 ± 1.05	—	—	—
Animal tallow, 4%	2.32 ± 0.34	33.25 ± 2.10	53.48 ± 2.11	10.97 ± 1.34	—	—	—
Animal tallow, 8%	2.18 ± 0.36	34.64 ± 0.39	55.43 ± 1.18	7.75 ± 0.61	—	—	—
Soybean oil, 4%	2.85 ± 0.51	29.72 ± 3.24	52.58 ± 1.46	14.85 ± 2.61	0.75 ± 0.29	—	—
Soybean oil, 8%	1.07 ± 0.80	21.83 ± 2.24	44.04 ± 4.40	33.06 ± 6.15	1.60 ± 0.21	—	—
Rapeseed oil, 4%	1.45 ± 0.48	26.51 ± 1.35	49.60 ± 4.29	11.65 ± 3.55	0.96 ± 0.32	5.10 ± 0.39	4.71 ± 0.33
Rapeseed oil, 8%	0.96 ± 0.57	17.36 ± 1.00	48.47 ± 2.94	17.73 ± 5.06	1.34 ± 0.26	8.06 ± 0.88	6.08 ± 0.98
Sunflower seed oil, 4%	3.27 ± 0.20	28.44 ± 0.97	51.42 ± 2.22	16.13 ± 3.29	—	—	—
Sunflower seed oil, 8%	1.30 ± 0.32	19.05 ± 1.00	36.69 ± 5.90	41.36 ± 7.28	—	—	—

[1] Figures preceded by ± sign are standard errors of the mean.

fractions supplied by the fat source. Inclusion of 8% of soybean oil or sunflower seed oil in the ration caused a marked decrease in the palmitic-palmitoleic acid ($C_{16} + C_{16:1}$) fraction of the adipose tissue with a simultaneous increase in the linoleic acid fraction. The presence of substantial portions of eicosenoic ($C_{20:1}$) and erucic ($C_{22:1}$) acids in adipose tissue of chicks receiving dietary rapeseed oil is further evidence for an apparent direct deposition of some unaltered dietary fatty acids in body fat stores. The influence of rapeseed oil on body fat of fowl had been reported by Chomyszyn ('55). He observed that, after administering 40 ml of rapeseed oil per day for 4 successive days, the subcutaneous and internal fat of young geese assumed the characteristics of rapeseed oil (iodine number, M.P. and odor).

The influence of dietary fatty acids on the carcass fat is not a unique occurrence. Horlich and O'Neil ('58) and Feigenbaum and Fisher ('59) observed that fatty acids of egg yolk could also be altered by degree of unsaturation of dietary fatty acids and Beare et al. ('61) showed that the milk fat from rats fed rapeseed oil contained eicosenoic and erucic acids, although in lesser quantities than present in the original oil.

The results of the present study indicate that the composition of the dietary fat may be an important consideration in the production of chicken carcasses for human consumption. Change in the physical characteristics of the carcass fat, and influence on cooking loss, development of rancidity and off-flavors due to various dietary fats are factors that require further study.

SUMMARY

Chickens were fed from zero to 8 weeks of age, rations containing 4 or 8% of animal tallow, soybean oil, rapeseed oil or sunflower seed oil. As compared with a ration with no added fat, only the inclusion of 4% of animal tallow in the ration failed to significantly increase weight gain. In general, all fat additions markedly improved efficiency of feed utilization. The use of equivalent levels of animal tallow, rapeseed oil and sunflower seed oil resulted in ration metabolizable energy values which were approximately equal. However, the metabolizable energy content of rations containing soybean oil was significantly higher than that of rations containing comparable levels of other fat sources. Fatty acid analysis by gas chromatography showed that the composition of chick adipose tissue reflected that of the dietary fat. The presence of substantial portions of eicosenoic and erucic acids in adipose tissue of chicks receiving dietary rapeseed oil provides additional evidence for an apparent direct deposition of some unaltered dietary fatty acids in the body fat stores.

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Factors Affecting the Metabolizable Energy Content of Poultry Feeds

2. VARIABILITY IN THE M.E. VALUES ATTRIBUTED TO SAMPLES OF TALLOW, AND UNDEGUMMED SOYBEAN OIL

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A KNOWLEDGE of the available energy content of poultry feeds is essential if the most economical rations are to be formulated. The question arises as to which method of describing available energy is to be employed. Practical difficulties, associated with the excretion of both feces and urine *via* a common cloaca, prevent the derivation of precise digestibility data. Axelsson (1939) and Halnan (1951) have argued strongly against the use of net energy values, and Titus (1955), Davidson *et al.* (1957) and Hill and Anderson (1958) have found productive energy data to be unsatisfactory. It is, therefore, understandable why many poultry nutritionists have turned to the evaluation of feeds in terms of metabolizable energy (M.E.).

It is possible that M.E. values are subject to certain intrinsic errors although there is little published evidence to either support or refute this hypothesis. Matterson *et al.* (1958) have demonstrated that the M.E. value for a sample of corn when fed alone was significantly lower than when fed at the expense of cellulose as part of a basal diet. Wilder *et al.* (1959) obtained M.E. values for certain fats which were greater than the gross energy values, while Sibbald *et al.* (1959, 1960) have shown that the M.E. value for corn varied when the basal diets, with which it was combined, differed in composition. These findings suggest that the 'associative effects of feeds,' observed in ruminant nutrition (Mitchell, 1942), may also occur in poultry nutrition.

The present report concerns an experiment designed to determine whether the M.E. values of tallow and undegummed soybean oil are additive. The influences of the protein level of the basal diets with which the fats were combined, and of the levels at which the fats were included in the test diets, were also determined. Additional information concerning the weight changes and feed utilization of the birds on the various diet treatments was collected and forms the subject of a subsequent report (Sibbald *et al.*, 1961).

EXPERIMENTAL PROCEDURES

Day-old male chicks (Barred Rocks and Columbian Rock×Rhode Island Reds) were placed in battery brooders where they received a chick starter diet (Basal, Table 1) until they reached the age of two weeks and four days. At this time they were individually weighed, divided into groups having a weight spread of 5 gm., and distributed between 56 pens until each pen contained 10 birds of each breeding. The experiment was conducted in a randomized block design with four replicated pens of chicks being subjected to each of the 14 diet treatments described in Table 2. The birds were fed the experi-

TABLE 1.—*Composition of the basal diets*

	Basal 1	Basal 2
	lbs.	lbs.
Ground corn (fine)	39.25	9.25
Ground wheat (fine)	15.00	15.00
Soybean oil meal (44% protein)	33.50	63.50
Dehydrated alfalfa (17% protein)	2.00	2.00
Meat meal (50% protein)	2.50	2.50
Fish meal (65% protein)	2.50	2.50
Dried whey (55% lactose)	2.50	2.50
Ground limestone	1.25	1.25
Dicalcium phosphate	1.00	1.00
Iodized salt	0.25	0.25

The following micronutrients were added to each 100 lb. of diet: manganese sulphate (75% $MnSO_4$) 9.0 gm., zinc oxide (80% ZnO) 3.0 gm., vitamin A supplement (10,000 I.U./gm.) 22.7 gm., vitamin D_3 supplement (1,500 I.C.U./gm.) 22.7 gm., DL-methionine (feed grade) 22.7 gm., riboflavin (1 gm./oz.) 6.0 gm., D-calcium pantothenate (2 gm./oz.) 6.0 gm., niacin 1.0 gm., penicillin (10 gm./lb.) 22.7 gm., vitamin B_{12} (9 mg./lb.) 34.1 gm., choline chloride (25%) 34.1 gm., 3-nitro, 4-hydroxy-phenylarsonic acid (10%) 22.7 gm.

mental diets for a 3-day ration acclimatization period followed by a 7-day experimental period during which the weight changes and feed consumption of each pen of chicks were recorded. One-hundred gram samples of excreta were collected from each pen on the third, fifth, and seventh day of the experimental period; the three samples from each pen were pooled, freeze-dried, and ground. Samples of each of the diets, together with the excreta samples, were assayed for gross energy using a Parr Oxygen Bomb Calorimeter, nitrogen by the method of Kjeldahl (A.O.A.C., 1955), and chromium sesquioxide by the method of Czarnocki *et al.* (1960). The tallow and undegummed soybean oil used in this experiment were also sampled and assayed for gross energy, nitrogen, ether extract, free fatty acids, iodine value, titre, unsaponifiables, moisture, saponification value and fatty acid composition; the latter analysis involved the preparation of the methyl esters of the fatty acids and the absorption thereof on a column packed with ethylene glycol succinate and butanediol succinate, helium gas being used as the carrier.[1]

TABLE 2.—*Composition and analyses of the experimental diets*[1]

Diet no.	Basal 1	Basal 2	Tallow[2]	Undegummed soybean oil[2]	Gross energy	Protein
	%	%	%	%	Cal./gm.	%
1	100	—	—	—	3.92	24.4
2	90	—	10	—	4.36	21.6
3	90	—	—	10	4.41	22.0
4	90	—	5	5	4.44	21.3
5	80	—	20	—	4.90	19.6
6	80	—	—	20	4.98	20.2
7	80	—	10	10	4.99	19.0
8	—	100	—	—	4.00	34.0
9	—	90	10	—	4.48	32.0
10	—	90	—	10	4.51	28.8
11	—	90	5	5	4.54	30.6
12	—	80	20	—	5.00	27.9
13	—	80	—	20	4.99	28.4
14	—	80	10	10	5.08	28.4

[1] 0.3% chromium sesquioxide (Cr_2O_3) added to each diet.
[2] Analysis of fat products.

	Grade B animal tallow	Undegummed soybean oil
Gross energy Cal./gm.	9.32	9.40
Protein %	0.1	0.0
Ether extract %	94.4	99.2
Moisture %	0.31	0.0
Free fatty acids %	4.8	0.62
Iodine value	56.4	132.1
Titre °C.	40.8	21.7
Unsaponifiable %	0.96	0.56
Saponification value	198.8	193.5
Fatty acid composition %		
C_8	Trace	—
C_{10}	0.05	—
C_{12}	0.08	—
C_{14}	2.50	Trace
C_{14} (Double bond)	0.46	—
C_{16}	0.18	—
C_{16}	26.9	11.3
C_{16} (Double bond)	3.3	0.26
C_{17}	0.35	Trace
C_{18}	17.4	3.9
C_{18} (Double bond)	41.5	27.2
C_{18} (2 Double bonds)	7.5	49.8
C_{18} (3 Double bonds)	0.11	7.5
C_{20}	—	0.04

Corrected M.E. values for the diets and for the fats were calculated by the methods of Sibbald *et al.* (1960) with the modification that the correction factor for retained nitrogen of 8.22 Cal./gm. as proposed by Hill and Anderson (1958) was replaced with the value of 8.73 Cal./gm. advocated by Titus *et al.* (1959).

The two basal diet treatment groups were employed as controls for the derivation of M.E. values for the fats which were combined with them to produce the 12 test diets. These latter 12 diets comprised a factorial arrangement consisting of 3 fats (tallow, undegummed soybean oil,

[1] We are indebted to Canada Packers Limited, Toronto, Canada, who conducted most of the fat analyses.

and a 50/50 mixture of the two), 2 fat levels (10 and 20%) and 2 basal diets of different protein content (24.4 and 34.0%). Analysis of variance was used to partition the total treatment variation into portions appropriate to individual degrees of freedom (Table 4) (Snedecor, 1956). The resulting mean squares were tested for significance by the tables of the studentized form of the extreme mean square test due to Nair (1948).

RESULTS AND DISCUSSION

The results of the chemical analyses of the 14 diets and of the 2 fats are appended to Table 2. Two interesting features are illustrated by these data; firstly it will be noted that the animal tallow had a much higher titre (40.8°C.) than did the undegummed soybean oil (21.7°C.), secondly it is apparent that the tallow contained less unsaturated fatty acids than did the soybean oil. This latter point is demonstrated in a gross manner by the iodine values of 56.4 and 132.1 for the tallow and soybean oil respectively; the fatty acid analysis confirms the gross observation while providing more detailed information of the nature of the difference. Palmitic and stearic acid account for approximately 44% of the fatty acids of the tallow and 15% of those of the soybean oil; tallow also contained more oleic acid than did the soybean oil (41.5 *versus* 27.2%) but was much poorer in linoleic (7.5 *versus* 49.8%) and linolenic (0.1 *versus* 7.5%).

The corrected M.E. values for the fats as derived from the 12 test diets are presented as means in Table 3. Examination of these data with respect to the protein levels of the basal diets with which the fats were combined shows that in all but one instance (20% undegummed soybean oil) the M.E. values were larger when the 34.0% protein basal was used than when the 24.4% protein basal was employed. Their averages 8.11 and 7.75 Cal./gm. respectively were found to be significantly different ($P<0.01$). The data of Table 3 suggest that much of the difference in M.E. value between protein levels was derived from the tallow (6.63–7.24 Cal./gm.) with a lesser amount attributable to the mixed fat (8.21–8.62 Cal./gm.) and practically none resulting from the undegummed soybean oil (8.44–8.48 Cal./gm.). This interaction of protein level×kind of fat failed to reach statistical significance. These data also suggest an interaction of protein level×fat levels where the M.E. values increase with increased fat level with the 24.4% protein basal (7.59–7.92 Cal./gm.) but decrease with increased fat level with the 34.0% protein basal (8.18–8.02 Cal./gm.). An F-test (Table 4) revealed that this interaction was not statistically significant. The three-factor interactions of basal diet protein level×kind of fat×fat level also failed to reveal statistically significant differences. It would therefore appear that the level of protein in the basal diets exerted a significant influence upon the M.E. values attributed to the fats with which they were combined.

TABLE 3.—*The metabolizable energy values*[1] *of tallow, undegummed soybean oil, and a 50/50 mixture of the two fats*

	Protein level of basal diet			
	24.4%		34.0%	
	Fat level			
	10%	20%	10%	20%
Kind of fat				
Tallow	6.02	7.24	6.79	7.69
Soybean oil	8.65	8.22	8.94	8.02
Mixed fat	8.11	8.31	8.82	8.41

[1] The standard error of each of these values, which are means of 4 determinations, is 0.16 Calories /gm.

A study of the data with respect to the types of fat indicates that there was little difference between the overall M.E. value for the undegummed soybean oil (8.46

TABLE 4.—*Orthogonal analysis of variance of the M.E. values attributed to the fats*

Source of variation	Degrees of freedom	Mean square
Replication	3	0.1508
Protein level of basal (P)	1	1.4981**
Tallow *vs.* soy oil+mixed fat (T)	1	24.0200**
Soy oil *vs.* mixed fat (S)	1	0.0144
Fat levels (F)	1	0.1027
P×T	1	0.3876
P×S	1	0.2664
P×F	1	0.6816
F×T	1	5.6743**
F×S	1	0.6612
P×F×T	1	0.0345
P×F×S	1	0.0072
Experimental error	33	0.0978
Total	47	

** Significant at the 1% level.

Cal./gm.) and that for the mixed fat (8.41 Cal./gm.); however, both these values were markedly higher than that of the tallow (6.94 Cal./gm.). Statistical support for these observations is presented in Table 4. Further investigation shows that the M.E. values of the undegummed soybean oil and mixed fat were not uniformly higher than those for the tallow but differed not only between basal diet protein levels but also between fat levels. As stated above the protein level×kinds of fat interactions were not significant. The interaction: fat level×undegummed soybean oil *versus* mixed fat also failed to demonstrate statistical significance. The interaction: fat level×tallow *versus* undegummed soybean oil+mixed fat, which is apparent in the data of Table 5, proved to be highly significant ($P<0.01$). Thus although the average M.E. values for all fats did not differ greatly between levels of dietary inclusion (10%=7.89, 20%=7.98 Cal./gm.) it is apparent that the M.E. value of tallow increased when its level of use in the diet increased from 10 to 20% (6.40–7.47 Cal./gm.) while the value of the undegummed soybean oil+mixed fat decreased (8.63–8.24 Cal./gm.). Similarly the lower M.E. value of the tallow (6.94 Cal./gm.) relative to that of undegummed soybean oil+mixed fat (8.44 Cal./gm.) derives primarily from the data relative to the 10% level of dietary inclusion. These findings demonstrate that not only were the M.E. values of the fats not additive but also that the level at which a fat is included in the diet can exert a profound influence upon the M.E. value attributed to that fat.

TABLE 5.—*The interaction between fats within fat levels*

	Tallow	Undegummed soybean oil+mixed fat	Average
	Cal./gm.	Cal./gm.	Cal./gm.
10% fat	6.40	8.63	7.89
20% fat	7.47	8.24	7.98
Average	6.94	8.44	

If the M.E. values of the fats were additive then the M.E. content of the mixed fat would be equivalent to the mean of the values for the tallow and the undegummed soybean oil and would be either significantly, or not significantly, different from both of them; it should certainly not be significantly different from the value for the tallow and not different from the undegummed soybean oil. It is apparent from the findings of this experiment that by combining tallow with an equal weight of undegummed soybean oil the M.E. content of the tallow, and/or the soybean oil, was greatly increased. As approximately 90% of the gross energy of the undegummed soybean oil was metabolized even in the absence of tallow it seems probable that an increased utilization of the gross energy of the tallow was the reason for the high M.E. content of the mixed fat.

This latter conclusion has considerable economic significance. If it is assumed that the M.E. value of the undegummed

soybean oil does not change when it is combined with tallow then it is possible to calculate the increase in the M.E. attributed to the tallow resulting from the presence of the soybean oil.

Viz:

M.E. contributed to the mixed fat by tallow

$= [\text{M.E./gm. mixed fat} - (0.5 \times \text{M.E./gm. soybean oil})] \times 2 \text{ Cal./gm.}$

M.E. gain/gm. of tallow in mixed fat

$= (\text{M.E./gm. tallow in mixed fat} - \text{M.E./gm. tallow}) \text{ Cal.}$

Such values have been calculated on a per lb. basis and are presented in Table 6. These data, together with those of Table 3, make it possible to determine the value of undegummed soybean oil when the price of tallow is known.

It would seem possible that some factor, or factors, present in the soybean oil gum, possibly phospholipids, allowed increased utilization of the tallow. Renner and Hill (1958) have reported that neither palmitic nor stearic acid contribute any M.E. to the chick and this could explain the difference between the M.E. values of the tallow and undegummed soybean oil for the tallow contained a much larger proportion of these fatty acids than did the soybean oil. The M.E. value of the mixed fat is such as to suggest that at least a portion of these fatty acids was utilized and it is possible that in the presence of soybean oil gums palmitic and/or stearic acid become partially available. The observed synergism between animal tallow and undegummed soybean oil would appear to warrant further investigation.

TABLE 6.—*The M.E. gain of tallow when combined with an equal weight of undegummed soybean oil*

	24.4% protein basal	34.0% protein basal	Mean
	Cal./lb.	Cal./lb.	Cal./lb.
Level of fat in diet			
10%	708	866	789
20%	520	503	512
Mean	612	690	644

SUMMARY

The metabolizable energy content of tallow, undegummed soybean oil, and a 50/50 mixture of these two fats, when fed at levels of 10 and 20%, has been determined. Basal diets containing respectively 24.4 and 34.0% protein were employed. It was found that the level of protein in the basal diet exerted a highly significant effect upon the utilization of the energy of the fats. The level at which the fat was included in the diet influenced the M.E. value of the tallow but not that of either the undegummed soybean oil or the mixed fat. The M.E. value attributed to tallow was significantly lower than those of the soybean oil and the mixed fat; the M.E. values of the two latter materials did not differ significantly. The M.E. values attributed to tallow and undegummed soybean oil were not additive suggesting that there was some factor present in the undegummed soybean oil which allowed increased utilization of the energy of the tallow.

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The Effect of Fats and Fatty Acids in Chick Rations

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RECENT studies by Sielder and Schweigert (1953), Yacowitz (1953), Sunde (1954a), and Runnels (1955) indicated that the use of animal fats in poultry rations improved the utilization of the feed for broilers. Sunde (1954b) and Carver *et al.* (1954) reported that a hydrogenated fat or stearic acid would not improve feed utilization, but that all other fats tested would improve the utilization of the feed fed to young chicks. Both workers reported that the saturated fats were not absorbed by the digestive tract, and therefore offered very little nutritionally.

Biely and March (1954) reported that in both chick and poult rations the addition of fat may be advantageous when relatively high levels of protein are fed. Siedler *et al.* (1955) reported that 3 or 6 percent fat or 3 percent free fatty acids did not decrease the rate of gain and were utilized efficiently.

EXPERIMENTAL PROCEDURE

Day-old chicks of both sexes, the progeny of New Hampshire males and Single Comb White Leghorn females, were used except in experiments 6, 10 and 11. In experiment 6, New Hampshire chicks obtained from a commercial hatchery were used. In experiment 10, cross bred male chicks of the above cross were used. They were 31 days old when placed on experiment. In experiment 11, White Plymouth Rock chicks obtained from a commercial hatchery were used. Twenty-five chicks were used per group except where indicated. In experiments 6 and 11, twenty chicks were used per group. In experiments 7 and 8, the groups fed the linolenic, linoleic and butyric acid contained only 13 or 15 chicks because of the cost of these materials. All experiments were conducted in standard type electric batteries with raised wire floors. In experiments 9 to 11 all the birds had access to both types of feed in each battery at all times. When duplicate groups were set up, the diets were alternated on each side of the battery. This would eliminate the light factor, and also the bird's desire to be near or away from the most activity. One feeder contained the added fat, and the other the feed with no added fat. The deck immediately below that one had the two types of feed reversed. Once during the experimental period the feeders were switched to see how long it took the birds to adjust to the new conditions. The basal diets fed in all experiments are shown in Table 1. Additions were made in such a way as to keep the protein level constant. This was done by decreasing the corn and increasing the soybean oil meal.

Tenox II* was added at the level of .05 percent to all the fats and fatty acids used except the stearic acid and the hydrogenated fats. The time required for feed

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The author is indebted to the Darling Manufacturing Co., Chicago, Illinois for the yellow grease, brown grease, prime tallow, and the No. 1 tallow used in this study.

* This mixture contains 20 percent butylated hydroxyanisole, 6 percent propyl gallate, 4 percent citric acid, and 70 percent propylene glycol.

TABLE 1.—*Basal diets*

	A (gms./kg.)	B
Ground yellow corn	450	555
Soybean oil meal (solvent)	300	350
Wheat bran	50	
Wheat middlings	50	
Alfalfa meal	50	30
Steamed bone meal	30	
Ground limestone	—	12.5
Di-calcium phosphate	—	17.5
Vitamin D_2 (1,500 D/gm.)	—	.5
Granite grit	10	
Chick size oystershell	20	
Iodized salt	5	2.5
Feeding oil (300 D-1,500A)	2	
Fish solubles	30	30
Vitamin B_{12} and antibiotic feed supplement*	1	1
DL-Methionine	0.5	0.5
$MnSO_4 \cdot H_2O$	0.22	.25
Ca. pantothenate mgs./kg.	5	22
Niacin mgs./kg.	10	35
Riboflavin mgs./kg.	3.2	7

To Diet B were added the following vitamins in mg. per Kg. of ration: thiamine HCl 4, pyridoxine HCl 7, d biotin .2, menadione .8, i-inositol 1,000, para-amino-benzoic acid 100, alpha tocopherol acetate 5, and folic acid 2.

* Each pound equivalent to the activity of not less than 3 milligrams of vitamin B_{12} (L. L. elution assay) and 2 grams procaine penicillin.

passage was determined by feeding 500 milligrams of carmine in a zero size capsule and determining the time required for the red dye to be voided. The chicks were four weeks old when this test was performed. Ten birds from each group were used for each measurement.

The crude tall oil† material contains about 41–52 percent rosin acids and 46–52 percent fatty acids. It is a product obtained from woodpulp manufacturing especially of pine. About 180–300 pounds of this material are produced per ton of woodpulp. The tall oil is a distilled product obtained from the crude tall oil. It contains about 61–71 percent fatty acids and 25–30 percent rosin acids. The fatty acid fraction contains about 45 percent oleic acid, 48 percent linoleic acid, and 6–7 percent palmitic acid. The rosin acid fraction was concentrated from the crude tall oil by removing as much as possible of the tall oil. The crystalline materials contain about 90 percent rosin acids. The rosin acid fraction is composed of about 30–40 percent abietic acid and 10–20 percent neoabietic acid. Other rosin acids are also present in smaller amounts.

† Ligro-produced by the West Virginia Pulp and Paper Company, Charleston, South Carolina.

TABLE 2.—*The effect of different grades of fat on growth and feed conversion*

Experiment 1

Additions to basal A	4 wk. wts. (gms.)	Gm. feed / Gm. wt. 4 wks.	Time of feed passage (minutes)	
None	292	2.02	146	132
None	288	2.17		
5% White grease	324	1.79		
5% Yellow grease	313	1.95		
5% Brown grease	298	1.93		
5% Prime tallow	321	1.97	126	144
2.5% Prime tallow	300	1.96		
5% No. 1 tallow	322	1.93		
5% Commercially stabilized fat*	293	1.94		
5% Hydrogenated fat†	289	2.03		

* Sta-fat—Darling Manufacturing Co., Chicago, Illinois.
† Hydropoid—Bowman Feed Products, Inc., Holland, Michigan.

The feces samples were collected on the second and forth week as indicated in the tables. The samples were weighed and the fat and free fatty acid analyses conducted according to the method of Saxon as described by Hawk, Oser and Summerson (1947).

RESULTS AND DISCUSSION

The results of the first experiment are shown in Table 2. The addition of five percent choice white grease, yellow grease, brown grease, prime tallow, No. 1 tallow, a commerically stabilized fat or hydrogenated fat‡ did not decrease the growth rate. In most instances an increase in the rate of growth was observed. All the supplements to the basal diet except the hydrogenated fat improved the feed utilization (gms. feed/gm. weight). These

‡ Hydropoid-Bowman Feed Products, Inc., Holland, Michigan.

TABLE 3.—*The effect of different grades of fat on growth and feed conversion*

Experiment 2

Additions to basal A	4 wk. wt. (gms.)	Gms. feed / Gm. wt. (4 wk.)
None	305	2.04
None	313	2.08
5% White grease	297	1.90
5% Yellow grease	302	1.96
5% Brown grease	294	2.02
5% Prime tallow	289	2.07
5% No. 1 tallow	297	1.91
5% Commercially stabilized fat*	311	1.89
5* Hydrogenated fat†	311	2.05
2.5% Hydrogenated fat†	305	2.18

* Sta-fat—Darling Manufacturing Co., Chicago, Illinois.

† Hydropoid—Bowman Feed Products, Inc., Holland, Michigan.

differences were repeated in other experiments. The possibility existed that the fat might decrease the rate of the passage of the feed through the intestinal tract. Munson *et al.* (1950) reported that dextrinized corn starch increased the time of excretion over that observed when either sucrose or lactose was used as the carbohydrate in rations for chicks. The added prime tallow did not increase the time required for the passage of the feed through the tract (Table 2). This extremely rapid passage of food through the young chick emphasizes even further the importance in poultry feeding of having all the essential nutrients present in proper amounts at all times.

Table 3 shows the data of another experiment conducted in about the same manner as experiment 1. The prime tallow was tested only at the 5 percent level, but the hydrogenated fat which was ineffective in improving feed utilization in experiment 1 was tested at both the 2½ and 5 percent levels. Again it did not decrease the growth rate, but was ineffective at either level at improving feed utilization. All other supplements except the prime tallow improved feed utilization in this experiment. In other experiments not reported here prime tallow has been effective in improving feed utilization. Aitken, Lindblad and Hunsaker (1955) also reported on the effects of tallow on feed efficiency. These experiments show that these grades of fats can be used effectively except the hydrogenated fat. This has been reported previously by Sunde (1954b), Carver *et al.* (1954), Donaldson, Combs and Romoser (1954), and Siedler, Scheid and Schweigert (1955). Five or 2½ percent fat was used in these experiments. The level of fat that will be most economical will depend on the price of the fat and the price of the grains. Yacowitz and Chamberlin (1954) fed levels from 1.5 to 3.0 percent. On floor litter 1.5 percent fat improved feed efficiency 7 percent as compared to 3.1 percent with 3 percent fat. In batteries, however, 3 percent was better than 1.5 percent. The work from this laboratory has suggested that levels of fat of as high as 10 to 22 percent (Sunde, 1955; Leong *et al.*, 1955) increased the feed efficiency even further. Combs and Romoser (1955) have also fed 15 percent with an increase in efficiency.

Table 4 shows the results of feeding 5 and 10 percent white grease, 5 percent oleic acid and 5 percent hydrogenated fat. None of the supplements decreased the

TABLE 4.—*The effect of fats and fatty acids on body weight, feed conversion, feces fat and free fatty acids in the feces of chicks*

Experiment 3

Additions to basal A	4 wk. wts.	Gms. feed / Gm. wt	% fat in dry feces	% FFA of total fat
None	281	2.14	1.18	65
None	325	2.09		
5% white grease	311	1.92	3.17	64
10% white grease	289	1.93	5.80	55
5% Oleic acid	292	1.96	3.68	61
5% Hydrogenated fat†	309	2.21	9.27	81

† Hydropoid—Bowman Feed Products, Inc., Holland, Michigan.

growth rate. Again the addition of 5 percent hydrogenated fat failed to improve the utilization of the feed. All other supplements improved this utilization. An attempt was made to determine the digestibility of the fat in the feed. The addition of the white grease increased the amount of fat in the dry feces by 2 to 5 times depending upon the level. This was slightly lower in subsequent experiments. Five percent oleic acid increased the fat in the feces about the same as the 5 percent white grease. The amount of fat in the feces resulting from the feeding of the hydrogenated fat increased about 8 to 9 times over that of the basal diet. This indicates that the fat was not utilized to any appreciable extent. An attempt was made to determine the amount of the ether soluble fraction that was present as free fatty acids. The feces from the group fed the hydrogenated fat contained 82 percent free fatty acids. This suggests that the glycerol portion of the molecule was removed and that the free fatty acids were not absorbed. The determination employs the milliequivalents of sodium used to titrate to the phenolphthalein end point and uses the 18 carbon fatty acid as a basis to determine the amount of free fatty acid present. Either the chicken has the ability to remove the glycerol portion of the fat and not the saturated fatty acid or the fat is broken down into smaller frag-

TABLE 5.—*The effect of fats and fatty acids on body weight, feed conversion, feces fat and free fatty acids in the feces of chicks*

Experiment 4

Additions to basal A	4 wk. wts.	Gms. feed / Gm. wt.	% fat* in dry feces
None	326	1.90	.44
5% white grease	336	1.89	1.96
5% Stearic acid	324	2.03	7.68
5% Oleic acid	326	1.77	1.78

* 2 week data.

TABLE 6.—*The effect of dispersing agents on body weight, feed conversion and feces fat of chicks*

Experiment 5

Additions to basal A	4 wk. wts.	Gm. feed / Gm. wt.	% fat in dry feces
None	330	2.05	2.63
5% white grease	350	1.77	1.57
.2% Sodium ligno-sulfonate*	342	2.07	1.00
.2% Sodium ligno-sulfonate* and white grease	358	1.98	3.18
5% Stearic acid	318	2.19	9.01
5% Stearic acid and sodium ligno-sulfonate	326	2.19	9.01
5% Stearic acid and .02% surfactant†	325	2.16	7.76
Practical broiler mash	345	2.01	1.70

* Marasperse N from Marathon Corporation, Rothschild, Wisconsin.
† Ethomid HT 25 from Armour Laboratories, Chicago, Illinois.

ments which have acid groups which can be titrated.

In experiment 4 (Table 5) stearic acid as well as oleic acid and white grease were included in the experimental design. This was done to determine the effect on absorption of the completely saturated fat. Again weights were not affected but feed utilization was not improved with stearic acid. The basal group was more efficient than average (Tables 2, 3, and 4). Oleic acid was especially effective in this experiment. The percent fat in the feces is about the same for the oleic acid as for the white grease, however, the stearic acid was not absorbed to any extent from the intestine.

Table 6 shows the results of an attempt to use a dispersing agent to improve the utilization of the stearic acid. Sodium ligno-sulfonate did not decrease the percent fat in the feces when added to a diet containing either white grease or stearic acid. The use of a surfactant was not very effective either. The only group to show any improvement in feed utilization was the one fed the white grease alone. Whether the sodium ligno-sulfonate actually decreased the feed efficiency is doubted since no effect one way or the other was exerted in any group except the one

TABLE 7.—*Effect of varying the energy in the basal diet on feed conversion and body weight at 10 weeks*

Experiment 6

	Wt. (gms.)		Gms. feed
	M	F	Gm. wt.
Basal diet (A)	1,515	1,367	3.01
Basal diet + 5% white grease	1,700	1,366	2.60
High energy basal (Diet B)	1,580	1,308	2.75
High energy basal+5% white grease	1,643	1,399	2.38

containing the white grease. Thus it appears these surface active materials do not increase the birds ability to remove long chain saturated fatty acids from the intestine.

The data presented here and the previous data from our laboratory were obtained using a diet of medium energy content. Many other laboratories have used higher energy diets. Table 8 shows the results of an experiment making a comparison between the two types of diets. The high energy diet was made by using largely corn and soybean oil meal with low levels of alfalfa meal and supplementing this diet with minerals and vitamins. Supplementing either diet with five percent white grease improved the growth rate slightly at 10 weeks. This has been true in several of our trials to 10 weeks, Sunde (1954a). The 10 weeks' data of Pepper *et al.* (1953), Siedler *et al.* (1955) and Runnels (1955) also suggest this same effect. It is of interest that the addition

TABLE 8.—*The effect of fatty acids on body weight and feed conversion in chicks*

Experiment 7

	Wt. (gms.)	Gms. feed / Gm. wt.
Basal diet A	280	2.21
Basal+5% oleic acid	276	1.95
Basal+5% stearic acid	294	2.08
Basal+5% linoleic acid	281	1.99
Basal+5% linolenic acid	275	1.96
Basal+5% butyric acid	258	2.00
Basal+5% white grease	277	1.88

TABLE 9.—*The effect on chicks of tall oil products, and fatty acids on body weight, feed conversion and feces fat*

Experiment 8

	Wt. (gms.)	Gms. feed / Gm. wt.	% fat in dry feces
Basal diet A	302.2	2.04	1.12
	312.5	2.03	1.20
Basal+5% crude tall oil	155.8	3.05	—
Basal+5% tall oil	201.7	3.01	2.44
Basal+5% rosin acids	88.9	—	—
Basal+5% oleic acid	282.7	1.95	1.89
Basal+5% butyric acid	204.7	1.95	.94
Basal+5% linoleic acid	316.3	1.91	1.45
Basal+5% linolenic acid	327.3	1.89	2.14
Basal+5% white grease	290.1	1.92	1.27

of white grease to either type diet resulted in a similar improvement in feed utilization. The improvement was .41 pound of feed in one instance and .37 in the other. Perhaps the protein level was a bit more than adequate for each energy level.

Tables 8 and 9 show the results of feeding several fatty acids to chicks. Butyric was included to see what use the chickens would make of this short chain fatty acid. Butyric acid depressed growth in both experiments. Feed utilization was improved with this material. Oleic acid, linolenic or linoleic acid improved the feed utilization and did not affect growth.

When the crude tall oil was fed, the growth of the chicks was depressed when compared to groups receiving oleic, linolenic or linoleic acid. Tall oil reduced the growth rate in spite of its high free fatty acid content. The cause of this reduction is probably due to its rosin acid content. When the rosin acids fraction was fed at the five percent level, growth was depressed markedly. The growth depressing effect of the crude tall oil was intermediate between the high fatty acid fraction and the high rosin acid fraction. All three of these products reduced feed utilization probably because of their depressing effect on growth. It is of interest to notice that the percent fat in the dry feces is as high as with oleic acid. No determinations of fat were made on the feces of the rosin

TABLE 10.—*The results of cafeteria feeding of feed with and without added fat to chicks*

	Feed consumed in grams				Consumption ratio	
	Without fat		With fat			
	A	B	A[1]	B[1]	A[1]/A	B[1]/B
Experiment 9						
Time						
0–7 days	380	385	545	705	1:1.4	1:1.8
0–14 days	1,100	1,045	2,367	3,048	1:2.15	1:2.9
0–21 days	2,315	2,720	5,380	6,260	1:2.3	1:2.3
0–28 days	4,950	4,595	9,610	10,285	1:1.9	1:2.2
Experiment 10						
31–35 days	315	562	1,972	1,990	1:6.3	1:3.5
31–41 days	1,825	983	3,585	3,747	1:2.0	1:3.8
31–48 days	2,140	1,545	5,557	5,737	1:2.6	1:3.7
Experiment 11						
0–7 days	500		1,100		1:2.2	
0–10 days	965		1,930		1:2.0	
0–17 days	1,820		3,825		1:2.1	
0–22 days	2,640		5,000		1:1.9	

acid fed chicks or those fed the crude tall oil because of the severe growth depressing effect of those materials. If the rosin acids could be removed from these materials efficiently, this product might find a use in poultry feeds. At the present time the price of the distilled oil is about 6½ cents per pound in tank car lots.

The data on the fatty acids tested shows that the free fatty acids themselves are not harmful and that the free fatty acid content of an animal fat is not important. The free fatty acid content of a material, however, may give some indication as to the past history of the material, and therefore may be of value to the purchaser of inedible animal fats. This is true not because of the free fatty acid content, but because of other break down products it may contain.

Table 10 shows the results of three experiments set up in such a way as to obtain data on whether or not the birds prefer feed with added fat. The groups were set up in duplicate in all but experiment 11. The data show that light or general activity were not important since feed consumption figures of the duplicate groups were about the same. The groups were distributed in such a way as to eliminate this as a variable. In all groups, the birds preferred the feed with the added fat. When given access to both types of feed, they consumed about two times as much of the feed containing added fat as the feed with no added fat. This relationship held both for the chicks started at one day of age and at 31 days of age. The chicks started at 31 days of age had been on another experiment until the 28th day. No added fat was included in any of the rations used up until the 31st day. Thus it appears that battery chicks, at least, prefer feed with added fat when they have a choice. This does not mean that they will eat more of a feed with added fat when given only one feed, but rather that when given a choice they prefer the one with the added fat. We do not know if texture or color is the more important factor.

SUMMARY

Representative types of inedible animal fats have been fed to chicks. Choice white

grease, brown grease, prime tallow and No. 1 tallow were used in chick starting diets without deleterious effects at the five percent level. All these materials improved feed utilization. Oleic acid, linolenic and linoleic acid did not affect the growth rate and improved feed utilization. The incorporation of five percent hydrogenated fat or stearic acid in the diet did not improve the feed utilization. Apparently the chicks did not utilize the saturated long chain fatty acids provided by these materials.

A comparison of a medium and a high energy formula was made and the addition of fat to either diet resulted in about the same improvement in feed conversion. Fatty acid fractions of crude tall oil preparations were toxic to the chicks probably because of the rosin acids which they contained.

Chicks given access to feed with and without added fat ate about twice as much of the feed with the added fat.

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Investigation of Fatty Acids and Derivatives for Carcinogenic Activity[1]

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SUMMARY

Twenty-nine fatty acids and esters, lactones, and epoxy and peroxy compounds were tested for carcinogenic activity by repeated s.c. injections in mice.

Sarcomas at the site of injection were elicited with 12-hydroxystearic acid, methyl 12-hydroxystearate, 4-Ketostearic acid, stearohydroxamic acid, glycidyl laurate, glycidyl oleate, and *p*-nitroperoxybenzoic acid.

Sarcomas were also elicited in mice given injections of lower doses of stearic acid and γ-stearolactone. These unexpected findings could not be interpreted satisfactorily and raise serious questions regarding the interpretation of results with other compounds. Nevertheless, it does appear that weak carcinogens for the subcutaneous tissue of mice occur among these classes of chemical compounds.

INTRODUCTION

During the past decade, increased attention has been directed to the role of environmental factors in carcinogenesis. A large number of structurally varied and potentially active organic compounds are always present in the atmosphere from industrial waste products and fuels. Not only are some of these compounds intrinsically active biologically, but their photochemical and thermooxidation products may also be active, even more than their precursors.

All food fats contain varying quantities of polyunsaturated and monounsaturated fatty acid moieties, and therefore they are labile substances that are readily autoxidized. The initial products of autoxidation are hydroperoxides, which rapidly undergo conversion to numerous oxygen-containing species such as epoxides, aldehydes, ketones, hydroxy compounds, lactones, and a wide variety of other reactive and potentially toxic substances.

Oxidation products from fats and fatty foods, as well as those from the atmosphere, are adventitious sources of oxidation products. Inhalation of tobacco smoke represents a unadventitious and deliberate regimen for introducing oxidation products directly into man and provides another incentive for studying the carcinogenic activity of oxidation products. In addition, it is also of interest to ascertain whether the well-known fatty acids, the nutritional qualities and safety of which are taken for granted, have any carcinogenic effects.

Not only are we interested in the carcinogenicity of oxidation products as described above, but, perhaps more important, we are searching for functional group-biological activity relationships. In this paper we report a preliminary study of the carcinogenicity of 4 groups of compounds to determine their possible carcinogenicity when administered s.c. in mice. The results are not entirely clear and admittedly are only preliminary. Nevertheless, publication was considered desirable at this time because of the interest in these environmental factors and to provide information of possible value to other investigators.

Dickens *et al.* (6–11) and Van Duuren *et al.* (28, 30) have published studies on the carcinogenicity of epoxides, lactones, and peroxy compounds. Most of the compounds described in the current study had not been reported at the time we began our investigation.

MATERIALS AND METHODS

Source and Preparation of Compounds. The list of chemical compounds, their structure, and their molecular weight are given in Table 1.

Lauric (I), palmitic (II), oleic (III), and stearic (IV) acids were prepared by conventional crystallization and distillation methods from the purest commercial starting materials, as described by Swern and Jordan (24). 4-Ketostearic acid (V) was prepared by chromic acid oxidation of 4-hydroxystearic acid, according to the method of Willits *et al.* (34). 2-Hydroxystearic acid (VI) was prepared from 2-bromostearic acid by hydrolysis as described by Sweet and Estes (23). Methyl 12-hydroxystearate (IX) was prepared by conventional acid-catalyzed esterification of 12-hydroxystearic (VII) acid with methanol; the 12-hydroxystearic acid was purified by extensive recrystallization of the purest commercial grade. Methyl stearate (VIII) was prepared from stearic acid (IV) by esterification. 9(10)-Monohydroxystearic acid (X) was prepared from oleic acid, according to the method of Swern *et al.* (25). Stearohydroxamic acid (XI) was prepared from methyl stearate and hydroxylamine as described by Roe and Swern (17).

Givaudan-Delawanna, Inc., New York, N. Y., provided γ-valerolactone (XV), γ-octalactone (XVIII), γ-nonalactone (XIX), and γ-undecalactone (XX); XV was used as supplied and the others were purified by fractional distillation under vacuum and/or column chromatography. Eastman Organic

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Table 1

Compounds evaluated

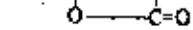
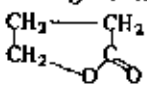
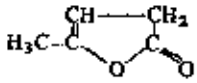
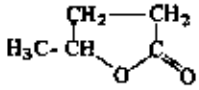
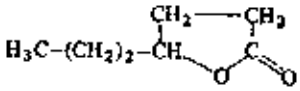
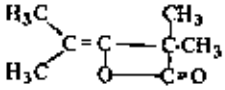
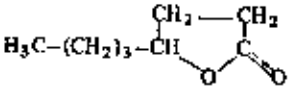
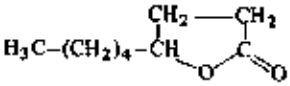
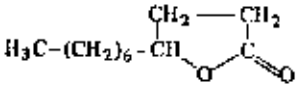
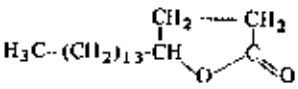
No.	Compound	Structure	Molecular weight
	Fatty acids and derivatives		
I	Lauric acid	$CH_3(CH_2)_{10}CO_2H$	200.3
II	Palmitic acid	$CH_3(CH_2)_{14}CO_2H$	256.4
III	Oleic acid	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$	282.5
IV	Stearic acid	$CH_3(CH_2)_{16}CO_2H$	284.5
V	4-Ketostearic acid	$CH_3(CH_2)_{13}-\underset{O}{C}-(CH_2)_2CO_2H$	298.5
VI	2-Hydroxystearic acid	$CH_3(CH_2)_{15}-\underset{OH}{C}HCO_2H$	300.5
VII	12-Hydroxystearic acid	$CH_3(CH_2)_5-\underset{OH}{C}H-(CH_2)_{10}-CO_2H$	300.5
VIII	Methyl stearate	$CH_3-(CH_2)_{16}-CO_2CH_3$	298.5
IX	Methyl 12-hydroxystearate	$CH_3(CH_2)_5\underset{OH}{C}H(CH_2)_{10}CO_2CH_3$	314.5
X	9(10)-Monohydroxystearic acid	$CH_3-(CH_2)_{7(8)}-\underset{OH}{C}H-(CH_2)_{8(7)}-CO_2H$	300.5
XI	Stearohydroxamic acid	$CH_3(CH_2)_{16}CONHOH$	299.5
	Lactones		
XII	β-Butyrolactone		86.1
XIII	γ-Butyrolactone		86.1
XIV	α-Angelicalactone		98.1
XV	γ-Valerolactone		100.1
XVI	γ-Heptalactone		128.1
XVII	2,2,4-Trimethyl-3-hydroxy-3-pentenoic acid lactone		140.2
XVIII	γ-Octalactone		142.2
XIX	γ-Nonalactone		156.2
XX	γ-Undecalactone		184.4
XXI	γ-Stearolactone		282.5

Table 1—continued

No.	Compound	Structure	Molecular weight
	Epoxy compounds		
XXII	*cis*-9,10-Epoxystearic acid	$CH_3(CH_2)_7-C(H)-C(H)-(CH_2)_7CO_2H$ (epoxide)	298.5
XXIII	*trans*-9,10-Epoxystearic acid	$CH_3(CH_2)_7-C(H)-C(H)-(CH_2)_7CO_2H$ (epoxide)	298.5
XXIV	Glycidyl laurate	$CH_3(CH_2)_{10}CO_2CH_2CH-CH_2$ (epoxide)	256.4
XXV	Glycidyl oleate	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2CH_2CH-CH$ (epoxide)	338.6
XXVI	Glycidyl stearate	$CH_3(CH_2)_{16}CO_2CH_2CH-CH_2$ (epoxide)	340.6
XXVII	*p*-Nitroperoxybenzoic acid	$O_2N-C_6H_4-CO_3H$	183.1
XXVIII	Peroxylauric acid	$CH_3(CH_2)_{10}CO_3H$	216.3
XXIX	Lauroyl peroxide	$CH_3(CH_2)_{10}-C(=O)-O-O-C(=O)-(CH_2)_{10}CH_3$	398.6

Chemicals, Rochester, N. Y., was the source of β-butyrolactone (XII), γ-butyrolactone (XIII), and 2,2,4-trimethyl-5-hydroxy-3-pentenoic acid lactone (XVII); XIII was used as supplied and the others were purified by fractional distillation under vacuum. K and K Laboratories, Plainview, N. Y., provided α-angelicalactone (XIV); it was purified by fractional distillation under vacuum (14). Fritzsche Brothers, Inc., New York, N. Y., was the source of γ-heptalactone (XVI), which was used as supplied. γ-Stearolactone (XXI) was prepared by isomerization of oleic acid by perchloric acid, followed by chromatography and recrystallization as described previously by Showell *et al.* (20). *cis* (XXII)- and *trans* (XXIII)-9,10-Epoxystearic acids were prepared by oxidation of oleic and elaidic acids, respectively, with peroxyacetic acid as reported by Findley *et al.* (13). Glycidyl laurate (XXIV), oleate (XXV), and stearate (XXVI) were prepared from epichlorhydrin and the salts of the corresponding acids, with the use of benzyltrimethylammonium chloride as catalyst according to the procedures described by Maerker *et al.* (15).

p-Nitroperoxybenzoic acid (XXVII) was prepared from *p*-nitrobenzoic acid and 90% hydrogen peroxide in methanesulfonic acid medium by the method of Silbert *et al.* (21). Peroxylauric acid (XXVIII) was prepared from lauric acid and 50% hydrogen peroxide in sulfuric acid by the method of Parker *et al.* (16). Lauroyl peroxide (XXIX) was prepared from lauroyl chloride and 50% hydrogen peroxide as described by Silbert and Swern (22).

Purity of all the compounds was determined by functional group analysis and microanalysis coupled with thin-layer, column, and gas-liquid chromatography (where applicable) and nuclear magnetic resonance spectroscopy. With the exception of *p*-nitroperoxybenzoic acid (XXVII), all compounds were greater than 98%, and usually greater than 99% pure. *p*-Nitroperoxybenzoic acid was 90% pure; the impurity was *p*-nitrobenzoic acid.

Tricaprylin used as a vehicle was of purity greater than 99%; it was purchased from Drew Chemical Co., Boonton, N. J.

Test Animals and Procedures. Female BALB/c (mammary tumor virus-free) mice were developed as a result of brother-sister matings of stock originally obtained from H. B. Andervont's colony. Upon reaching 2 months of age, they were divided by random distribution into groups of 10 to 16 animals each.

Female CFW (Swiss-Webster) mice, 2 months old, were obtained from Carworth, Inc., New York, N. Y. These randomly bred animals were arbitrarily placed in groups of 16 mice each following a 1-week quarantine after shipments were received.

The mice were housed in groups of 8 in plastic shoe-box type cages in air-conditioned quarters. Heat-treated absorbent cedar cubed wood (Ab-Sorb-Dri, Inc., Garfield, N. J.) with known moisture content and particle size was used for bedding. Teklad mouse diet pellets (purchased from Teklad, Inc., Monmouth, Ill.) and water were available *ad libitum*. Water bottles were treated weekly with a germicidal detergent (Mikro-Quat, Economics Laboratory, St. Paul, Minn.). The mice were transferred to clean cages twice a week.

The female mice were afforded no access to males at any time.

Injections were administered s.c. in the inguinal area; the mice received injections 3 times a week for 4 weeks. Materials administered daily or for periods of longer than 4 weeks were given in inguinal and axillary areas because of large subcutaneous pockets of unabsorbed oil. Steam-sterilized glass syringes fitted with 25-gauge needles were used, except for some instances when larger-bore needles were required because of the slurry-like nature of the material for injection. The vehicle for all injections was tricaprylin and the volume per injection was 0.1 ml.

In some instances ulcerations developed at the sites of injection, but they were too few in number to be adjudged as evidence of a reaction to the material administered.

Dosage was based on preliminary toxicological evaluations prior to the start of treatment.

All mice were weighed at the start of the experiments and at regular intervals throughout the ensuing period of observation of 18 to 24 months. No excessive weight losses or gains were found as compared with untreated control groups.

Attempts to transplant tumors from the sites of injection to other (BALB/c) mice were successful with 6 of 6 tumors, but this procedure was not continued routinely.

Mice were observed twice weekly for the appearance of subcutaneous tumors. Animals with tumors or those in poor condition were killed and autopsied. Suspected neoplasms and other grossly abnormal tissues were removed and fixed in buffered 10% formalin. All diagnoses were based on histological examination of sections stained with hematoxylin and eosin.

Four series of investigations were carried out from 1964 to 1967 utilizing 1245 female mice of BALB/c or Swiss strain. Smaller experiments with C3H male mice and strain A male mice are also recorded in the text.

RESULTS

Results of the 4 major series of investigations are given in Table 2, which indicates the conditions of administration, survival, and occurrence of malignant neoplasms for each group of mice. Table 3 summarizes the data of Table 2.

The conditions of the investigation during the first 2 years

Table 2

Detailed data on tests for carcinogenic activity

No.	Compound	Year	Dose/injection (mg)	Schedule (injection No./wk/total injections)	Total dose (mg compound/ml tricaprylin)	Mouse strain	Mice alive at month 0	6	9	12	15	18	Tumors by month of appearance: Subcutaneous sarcomas	Pulmonary tumor	Other tumors
						Controls									
	Tricaprylin	1964		2/104	0/10.4	S[a]	40	38	32	28	23	12		23	B(12), U(17, 19), C(12)
	Tricaprylin	1965		3/12	0/1.2	S	15	14	13	12	7	5		15, 18	B(22)
	Tricaprylin	1965		2/104	0/10.4	C	10	7	7	6	5	5	13	25	
	Tricaprylin	1966		3/10	0/1.0	S	29	29	25	20	6	4		24	
	Tricaprylin	1968		1/26	0/2.6	S	16	16	14	14					B(12)
	None	1965				S	24	23	23	22	17	13		24	
	None	1965				S	100	80	66	56	31	21	14	14, 17, 17, 17, 17	B(11, 14, 15, 17, 17, 17, 17, 17, 18, 18), C(14, 15)
	None	1965				S	47	45	43	13	8	3		18	B(11, 13, 16, 18)
	None	1965				C	26	31	29	27	23	12		23	L(6, 23, 23, 23)
	None	1967				S	32	23	23	14	13			12, 12, 12	
						Fatty acids and esters									
I	Lauric acid	1966	5.0	2/25	125/2.5	S	16	13	8	8	5	5	18	18	L(4, 5)
	Lauric acid	1966	1.0	3/12	12/1.2	S	15	15	13	13	9	8		23	L(23)
II	Palmitic acid	1966	5.0	2/25	125/2.5	S	16	10	8	8	6	5	8		B(18, 18), L(12)
	Palmitic acid	1966	1.0	3/10	10/1	S	16	16	15	8	8	6	19	19, 22	B(22)
III	Oleic acid	1966	0.5	2/33	11.5/2.3	S	16	11	11	8	5	4			B(9)
	Oleic acid	1966	0.1	3/10	1.0/1	S	15	15	14	9	5	1			
IV	Stearic acid	1964	1.0	2/82	82/8.2	C	10	8	8	8	8	7			
	Stearic acid	1964	0.5	2/114	57.0/10.4	C	10	10	10	10	10	9		21	Ad (21)
	Stearic acid	1964	0.05	2/114	5.7/11.4	C	10	9	8	6	5	4	6, 10, 10, 12	19	L(19)
	Stearic acid	1965	0.05	2/104	5.2/10.4	C	15	14	14	14	14	13		19	
	Stearic acid	1966	1.0	3/10	10/1	S	15	15	14	8	4	1			
	Stearic acid	1967	0.5	1/26	13/2.6	S	16	14	7	7	7	6			
	Stearic acid	1967	0.05	1/26	1.3/2.6	S	16	13	12	11	10	10			
V	4-Ketostearic acid	1966	1.0	2/50	50/5	C	15	13	13	11	6	0	10, 15		
VI	2-Hydroxystearic acid	1965	1.25	2/10.4	130/10.4	C	15	14	14	14	14	11		23, 23	
VII	12-Hydroxystearic acid	1965	1.0	2/80	80/8	S	15	14	10	8	8	6		14	L(11)
	12-Hydroxystearic acid	1965	0.05	2/80	4/8	S	15	14	13	7	2	0	11, 11, 11, 11, 12, 12, 13, 14, 15	11	L(11)
VIII	Methyl stearate	1967	5.0	1/26	130/2.6	S	16	16	16	16	14	11	15, 15	15, 15	
	Methyl stearate	1967	0.5	1/26	13/2.6	S	16	16	16	15	15				
IX	Methyl 12-hydroxystearate	1965	0.5	2/80	40/8	S	15	14	13	11	8	6	11, 13, 16	14	
	Methyl 12-hydroxystearate	1965	5.0	2/80	400/8	S	15	13	13	13	13	12	17	15	
X	4-Ketostearic acid	1966	1.0	2/50	50/5	C	15	13	13	11	6	0	10, 15		
XI	2-Hydroxystearic acid	1965	1.25	2/104	130/10.4	C	15	14	14	14	14	11		23, 23	

Table 2- continued

No.	Compound	Year	Dose/injection (mg)	Schedule (injection No./wk/total injections)	Total dose (mg compound/ml tricaprylin)	Mouse strain	Mice alive at month 0	6	9	12	15	18	Tumors by month of appearance: Subcutaneous sarcomas	Pulmonary tumor	Other tumors
	Lactones														
XII	β-Butyrolactone	1968	0.2	3/12	2.4/1.2	S	16	15	15	15			3		
XIII	γ-Butyrolactone	1967	0.005	3/12	0.06/1.2	S	16	14	12	12	11	11			
XIV	α-Angelicalactone	1968	0.15	3/12	1.8/1.2	S	16	15	15	15					
XV	γ-Valerolactone	1967	2.0	3/12	24/1.2	S	16	16	16	16	16	13			
XVI	γ-Heptalactone	1967	2.0	3/12	24/1.2	S	16	15	15	15	12	10			
XVII	2,2,4-trimethyl-3-hydroxy-3-pentenoic acid lactone	1968	0.2	3/12	2.4/1.2	S	16	16	16	16					
XVIII	γ-Octalactone	1967	12.0	3/12	144/1.2	S	16	16	15	13	12	8	12		
XIX	γ-Nonalactone	1967	15.0	3/12	180/1.2	S	16	15	15	15	11	8	14		
XX	γ-Undecalactone	1967	25.0	3/12	300/1.2	S	16	4	4	4	4				
XXI	γ-Stearolactone	1965	1.0	2/80	80/8	S	15	14	12	10	9	7	8, 17	10, 10	
	γ-Stearolactone	1965	0.5	2/80	40/8	S	15	13	10	9	5	3			U(17)
	γ-Stearolactone	1965	0.05	2/80	4/8	S	15	13	7	4	0		4, 6, 6, 8, 9, 9, 9, 9, 12	13	L(6, 9)
	γ-Stearolactone	1966	0.5	3/10	5.0/1	S	16	15	15	14	4	4		23	
	γ-Stearolactone	1966	0.05	3/10	0.5/1	S	16	15	15	15	7	7	23	23, 23, 23	U(23)
XXI	Stearolactone	1966	0.005	3/10	0.05/1	S	16	15	15	7	7	6	18		
	Stearolactone	1967	1.0	1/26	26/2.6	S	16	16	15	15	12	9	15	15, 17	B(17)
	Stearolactone	1967	0.005	1/26	1.3/2.6	S	16	16	15	15	12		18	15, 18	
	Epoxy compounds														
XXII	*cis*-9,10-Epoxystearic acid	1965	1.0	2/114	114/11.4	C	10	9	9	9	7	4	18	20	C(13)
	cis-9-10-Epoxystearic acid	1965	0.5	2/114	57/11.4	C	10	10	10	10	8	7	11		
	cis-9,10-Epoxystearic acid	1965	0.005	2/80	4/8	C	10	9	8	7	7	7	6	21	K(21)
	cis-9,10-Epoxystearic acid	1965	1.0	2/82	82/8.2	S	15	14	13	11	8	5			L(14)
	cis-9,10-Epoxystearic acid	1965	0.5	2/82	41/8.2	S	15	13	10	9	7	5	10, 18	17	G(17)
	cis-9,10-Epoxystearic acid	1965	0.005	2/82	4.1/8.2	S	15	15	15	15	13	10	16	17, 17	B(17), U(14)
XXIII	*trans*-9,10-Epoxystearic acid	1966	1.0	2/50	50/5	C	15	14	14	13	9	2	17		
XXIV	Glycidyl laurate	1964	0.005	2/62	3.1/6.2	C	4	4	4	3	2	2	10, 12		
	Glycidyl laurate	1965	0.1	2/80	8/8	S	15	12	12	10	3	0	12, 17	14, 16, 17	U(12)
	Glycidyl laurate	1965	0.005	2/80	0.4/8	S	15	14	8	6	4	0	6, 7, 7, 8, 8, 8, 9, 12, 15, 15, 15, 15	12, 13, 13, 15	
XXV	Glycidyl oleate	1965	0.25	2/104	26/10.4	C	15	13	10	7	5	4	10, 11, 11, 11, 11	17, 20, 20, 21	L(20)
XXVI	Glycidyl stearate	1964	10	2/66	660/6.6	C	12	12	11	11	10	5	23	23, 24	
	Glycidyl stearate	1967	0.1	1/26	2.6/2.6	S	16	16	16	14	13	4	16	15, 16	B(15)
	Glycidyl stearate	1967	0.005	1/26	1.3/2.6	S	16	16	15	15	13	11	16	16	
	Peroxy compounds														
XXVII	*p*-Nitroperoxybenzoic acid	1964	1.0	2/114	114/11.4	C	10	8	7	4	4	3			
	p-Nitroperoxybenzoic acid	1964	0.5	2/114	57/11.4	C	10	9	9	9	9	8	23		
	p-Nitroperoxybenzoic acid	1964	0.05	2/114	5.7/11.4	C	10	8	6	6	5	5	4, 7, 8		
	p-Nitroperoxybenzoic acid	1965	0.05	2/104	5.2/10.4	C	15	11	11	10	9	8	4		
	p-Nitroperoxybenzoic acid	1967	1.0	1/26	26/2.6	S	16	7	7	6	5	4			
	p-Nitroperoxybenzoic acid	1967	0.05	1/26	1.3/2.6	S	16	16	16	15	12		15	16	L(9), Sa(16, 16)
XXVIII	Peroxylauric acid	1965	5.0	2/80	400/8	S	15	13	10	10	5	5		17	B(8), L(6, 8, 17)
XXIX	Lauroyl peroxide	1965	0.05	2/104	5.2/10.4	C	15	15	15	15	15	14			

[a]S, Swiss-Webster; C, BALB/c; B, breast carcinoma; U, uterine carcinoma or sarcoma; C, cutaneous carcinoma; L, leukemia-lymphoma; Ad, adrenal carcinoma; K, kidney carcinoma; G, gastric carcinoma; Sa, salivary gland carcinoma.

dissolved s.c. injection in approximately the same site of the inguinal and axillary regions repeated 2 or 3 times each week for from 80 to over 100 injections. During this period 69 sarcomas were evoked in 366 mice given injections of experimental compounds. The yield of 18% seemed surprisingly high and included some unexplained responses to lower doses of presumably inactive compounds. Continual repeated injections also were hard on the mice and, logistically, to the technical personnel. During the latter 2 years, therefore, the injection schedule was reduced to 3 times/week for 12 doses, or once a week for 26 doses. Although in many instances the total dose overlapped the longer schedule, the yield of subcutaneous sarcomas dropped to 16 in 393 mice, or 4%. Other compounds were added,

Table 3

Summary of tests for carcinogenic activity

No.	Compound	Mouse strain	No. of mice alive at 6 months	Subcutaneous sarcomas	Pulmonary tumors	Breast cancers	Lymphomas	[illegible]
	Controls							
	Tricaprylin	S[a] and C	104	1	5	2	0	[illegible]
	No treatment	S and C	202	1	11	14	4	[illegible]
	Fatty acids and esters							
I	Lauric acid	S	28	1	2	0	2	[illegible]
II	Palmitic acid	S	26	2	2	3	1	[illegible]
III	Oleic acid	S	26	0	0	1	0	[illegible]
IV	Stearic acid	S and C	83	4	3	0	1	[illegible]
V	4-Ketostearic acid	C	13	2	0	0	0	[illegible]
VI	2-Hydroxystearic acid	C	14	0	2	0	0	[illegible]
VII	12-Hydroxystearic acid	S	28	9	2	0	2	[illegible]
VIII	Methyl stearate	S	32	2	2	0	0	[illegible]
VIX	Methyl 12-hydroxystearate	S	27	8	2	1	0	[illegible]
X	9(10)-Monohydroxystearic acid	C	13	1	1	0	0	[illegible]
XI	Stearohydroxamic acid	C	14	3	5	0	2	[illegible]
	Lactones							
XII	β-Butyrolactone	S	15	1	0	0	0	[illegible]
XIII	γ-Butyrolactone	S	14	0	0	0	0	[illegible]
XIV	α-Angelicalactone	S	15	0	0	0	0	[illegible]
XV	γ-Valerolactone	S	16	0	0	0	0	[illegible]
XVI	γ-Heptalactone	S	15	0	0	0	0	[illegible]
XVII	2,2,4-Trimethyl-3-hydroxy-	S	16	0	0	0	0	[illegible]
	3-pentenoic acid lactone	S	16	0	0	0	0	[illegible]
XVIII	γ-Octalactone	S	16	1	0	0	0	[illegible]
XIX	γ-Nonalactone	S	15	1	0	0	0	[illegible]
XX	γ-Undecalactone	S	4	0	0	0	0	[illegible]
XXI	γ-Stearolactone	S	117	15	11	0	0	[illegible]
	Epoxy compounds							
XXII	*cis*-9,10-Epoxystearic acid	S and C	70	6	5	1	1	[illegible]
XXIII	*trans*-9,10-Epoxystearic acid	C	14	1	0	0	0	[illegible]
XXIV	Glycidyl laurate	S and C	30	15	7	0	0	[illegible]
XXV	Glycidyl oleate	S and C	13	5	4	0	1	[illegible]
XXVI	Glycidyl stearate	S and C	44	3	5	1	0	[illegible]
	Peroxy compounds							
XXVII	*p*-Nitroperoxybenzoic acid	S and C	59	6	1	0	2	[illegible]
XXVIII	Peroxylauric acid	S	13	0	1	1	3	[illegible]
XXIX	Lauroyl peroxide	C	15	0	0	0	0	[illegible]

[a] S, Swiss-Webster; C, BALB/c.

however, so that no exact comparison of the sarcoma response during the 2 periods should be drawn.

Comparison of BALB/c mice and Swiss mice indicated similarity of subcutaneous response. Among 217 BALB/c mice, 28 sarcomas were elicited (12.6%); among 542 Swiss mice there were 57 sarcomas (10.5%).

Our analysis of the 29 compounds divided into 4 major types is as follows:

Controls. Among the 202 untreated and 104 vehicle controls, there were 2 subcutaneous sarcomas. This represents a frequency under 1%, and the appearance times were at 13 and 14 months of observation.

Pulmonary tumors, usually single, were found in [illegible] mice surviving 6 months (5.2%). Among 262 virgin S-W female mice 16 breast carcinomas occurred, a frequency of 6.1%, at an average age of 16.5 months. BALB/c mice developed no mammary tumors, but 4 of [illegible] had lymphomas.

The "background noise" of neoplasms in the mice used, therefore, was under 1% for subcutaneous sarcomas and under 10% for mammary and pulmonary tumors, practically all tumors appeared after 12 months of observation when the mice were 14 months old.

Fatty Acids and Esters. Eleven compounds were tested

between 1964 and 1967. Five compounds are considered negative, in that no sarcomas at the site of injection occurred with 3 compounds, and only 1 with the 2 others. Two compounds, methyl 12-hydroxystearate (IX) and 12-hydroxystearic acid (VII), produced sarcomas in 8 of 27 and 9 of 28 mice, respectively, and are classified as tentatively carcinogenic. With the latter compound, all sarcomas appeared at the lower dose of 4 mg over 80 weeks, and none at 80 mg. There was no significant increase in the number of lung tumors.

Two compounds, stearohydroxamic acid (XI) and 4-ketostearic acid (V), yielded 3 and 2 sarcomas in 13 and 14 mice, respectively. They are tentatively considered to have marginal carcinogenic activity.

A serious problem was encountered with stearic acid (IV). In the 1964 experiments, the group of 10 BALB/c mice receiving the lowest dose had 4 sarcomas. Three repeated experiments, 1 of which used approximately the same schedule, dose, and strain, were entirely negative. There was no increase in the number of lung tumors or other neoplasms. We have no explanation for this finding, except to note that all sarcomas occurred in 1 cage housing 8 animals. The total information available leads us to conclude that stearic acid is not carcinogenic under the conditions of our test. The aberrant result in 1 group, however, obviously poses a question with regard to the interpretation of responses with other chemicals.

The data suggest that several members of the 11 compounds tested should be regarded as possessing marginal carcinogenic activity for the subcutaneous tissue of mice.

Lactones. Of the 10 lactones tested, 9 were judged to be negative for carcinogenic activity, since no more than a single sarcoma at the site of injection was evoked, and there was no increase in other types of tumor. The absence of mammary tumors in 243 mice is worthy of note since, in comparison, 15 of 269 control animals developed breast cancer.

Four separate experiments were performed with γ-stearolactone (XXI); 3 are recorded in Table 2. In addition, 24 C3H male mice were given injections of up to 270 mg of the compound in sesame oil. Two mice developed sarcoma at the site of injection at 6 and 7 months, but only 2 of 6 mice given sesame oil also had sarcomas at 17 months.

The results recorded in Table 2 show that in the first experiments during 1965 11 sarcomas were evoked in 45 Swiss females at the site of injection. However, the largest number was seen in the group receiving the smallest dose, given in 80 injections over 40 weeks. In 2 repeated experiments in 1966 and 1967, only 1 sarcoma was found, although the injection schedule was considerably shorter. Ten of 117 mice that survived 6 months had pulmonary tumors, as compared with 13 in 269 control mice, a difference that is not significant.

Analysis of the 1965 groups of 15 mice given injections of γ-stearolactone (XXI) that yielded 9 sarcomas showed that they were housed in 2 cages, in which 6 occurred in 1 cage of 8 mice and 3 occurred in another of 7 mice. No explanation is apparent for these aberrant findings.

With the possible exception of γ-stearolactone, therefore, we conclude that the lactones tested were devoid of significant carcinogenic activity for the subcutaneous tissue of mice.

Epoxy Compounds. Five epoxy compounds were tested. Two compounds are tentatively classified as carcinogenic: glycidyl laurate (XXIV) and glycidyl oleate (XXV). These compounds yielded 14 sarcomas at the site of injection in 30 mice and 5 sarcomas in 13 mice, respectively. The number of lung tumors also seemed to be increased as compared with controls. With glycidyl laurate, the repeated experiments yielded sarcomas, but the largest number was obtained with the lowest dose.

Glycidyl stearate (XXVI) produced 3 sarcomas at injection sites with the highest dose and the longest schedule. *cis*-9,10-Epoxystearic acid (XXII) was used in strains BALB/c and Swiss; in 6 groups 1 was negative, whereas 4 showed single sarcomas and 1 had 2 sarcomas. The total of 6 sarcomas in 70 mice attracts attention, but epoxystearic acid is considered to be only marginally active.

Nevertheless, among 171 mice receiving the 5 epoxy compounds 30 sarcomas and 21 pulmonary tumors were evoked. It appears that this group of compounds contains carcinogens for the subcutaneous tissue of mice.

Peroxy Compounds. Of the 3 chemicals tested, 2 were negative on single tests. With *p*-nitroperoxybenzoic acid (XXVII), 6 sarcomas were elicited among 59 mice, without any increase in other types of tumors. The results suggest that this compound causes marginal carcinogenic activity in the subcutaneous tissue of mice.

Pulmonary Tumor Test of 3 Compounds. The carcinogenic activity of 3 compounds also was explored by the pulmonary tumor induction technique, as detailed for alkylating agents (18) and for carbamates (19). Strain A/He male mice, 2 months old, received 12 i.p. injections (3 times/week for 4 weeks) of 12-hydroxystearic acid (VII), γ-stearolactone (XXI), and glycidyl laurate (XXIV) dissolved in 0.1 ml tricaprylin. The total doses of 12-monohydroxystearic acid and of γ-stearolactone were 60 mg each; glycidyl laurate was 120 mg.

The mice were killed 20 weeks after the last injection. The lungs were fixed, and the typical pulmonary nodules were counted. Of 9 mice receiving 12-monohydroxystearic acid, 1 mouse had a single nodule and 2 had 2 pulmonary tumors each, for a mean of 0.6 lung tumors/animal. With γ-stearolactone, 13 of 15 mice were free of nodules in the lung and 2 mice had 1 and 2 nodules, respectively, or a mean of 0.2 lung tumor. Fourteen mice given injections of glycidyl laurate included 3 with single nodules, or a mean of 0.2 lung tumor. Comparison of these results with untreated mice shows that this frequency of lung tumors was within the spontaneous occurrence.

The tests, with the doses used, are considered as being negative for evidence of carcinogenic activity for the pulmonary tissue of strain A mice.

Interpretive Considerations. The conclusions derived from the findings of this study are indicated as tentative, since we are uneasy about the interpretation of the data. It was already noted that the experimental groups with more than 1 sarcoma represent experiments conducted during 1964 to

1965. The groups, therefore, received injections twice a week for 40 to 57 weeks.

It is most peculiar that the largest number of sarcomas appeared in groups that received smaller doses of the compounds. This is seen uniformly with 6 compounds injected at several dose levels:

Compound	Lower dose	Higher dose
Stearic acid (IV)	4/25	0/15
12-Hydroxystearic acid (VII)	9/15	0/15
Methyl 12-hydroxystearate (IX)	5/15	3/15
γ-Stearolactone (XXI)	9/15	2/32
Glycidyl laurate (XXIV)	11/15	4/19
p-Nitroperoxybenzoic acid (XXVII)	4/25	1/20
Total	42/110 (38%)	10/116 (9%)

We are at a loss to explain this and can only record it as an observation.

Another consideration involved the fact that in all groups of 15 or 16 mice the animals were housed in 2 cages throughout the experiment, although usually these 2 cages were kept next to each other, as well as to other cages. The distribution of multiple sarcomas in the 2 cages was as follows:

Compound	Cage 1	Cage 2
Stearic acid (IV)	4/8	0/2
12-Hydroxystearic acid (VII)	4/8	5/7
Methyl 12-hydroxystearate (IX)	1/8	2/7
Stearohydroxamic acid (XI)	2/8	1/7
γ-Stearolactone (XXI)	6/8	3/7
Glycidyl laurate (XXIV)	6/8	4/7
Glycidyl oleate (XXV)	3/8	2/7
p-Nitroperoxybenzoic acid	3/8	0/2
Totals	29/64 (45%)	17/46 (37%)

There is no indication, therefore, that sarcomas in several animals were limited to 1 of the 2 cages. The proportion of animals with sarcoma was similar in the 2 cages. At the same time there were at least 18 cages in which only 1 of up to 8 mice developed sarcoma. These observations would not support the idea that some transmissible infectious agent, viral or otherwise, was "lighted up" during the experiments.

DISCUSSION

Epidemiological observations have suggested possibilities that the occurrence of cancer, particularly of the gastrointestinal tract and of the liver, may be related to the presence of carcinogenic chemicals in diet. Identification of such chemicals in dietary constituents has concerned many workers during the past 30 years. During the period of the polycyclic hydrocarbons, especially in the 1940's, attention was directed to meat and meat products or vegetable oils subjected to high temperatures in cooking. This complex field, filled with irreproducible results, was critically viewed by Arffman (1). It led to particular emphasis upon work with cholesterol and its derivatives, another complex, unsure area that was reviewed by Bischoff (2) and Bischoff and Bryson (3).

The more recent approach to the problem of identifying possible carcinogens in dietary constituents stems from investigations on alkylating agents, another group of carcinogenic compounds. These simpler, rapidly reactive compounds with characteristic molecular features suggested possible mechanisms of biological interaction. Walpole *et al.* (32) in 1954 reported the induction of sarcomas at the site of injection in rats with β-propiolactone, adding this chemical configuration to the ethyleneimines and the epoxides as having carcinogenic import. Interest in this field was further stimulated by the discovery of aflatoxin, a potent carcinogen and chemically a member of the lactone family.

Among the more recent and comprehensive additions to the literature in this area of carcinogenesis have been those of Dickens *et al.* (6–12) and of Van Duuren *et al.* (26–31).

Dickens and Jones (9), using repeated s.c. injection in undefined rats, reported the appearance of sarcomas at the site of injection of β-propiolactone and other lactones. The chemicals were dissolved in arachis oil and were administered twice weekly for up to 61 weeks. No subcutaneous sarcomas were obtained with arachis oil alone. The yield of sarcomas with lactones was usually small, and the time of appearance usually well over 1 year, when only small groups of rats were still alive. In a series of subsequent publications (7, 10–12), more chemicals and crude derivatives were reported as actively carcinogenic on the basis of elicitation of sarcoma at the site of injection. With few exceptions, the yield of sarcomas was low and the tumors appeared after long periods of time.

Van Duuren *et al.* (26–31) have furnished more convincing evidence of carcinogenic activity among their series of lactones, epoxides, and peroxy compounds, since they used mice as well as rats and the cutaneous painting technique as well as subcutaneous injection. Among their compounds, several with suggestive weak carcinogenic activity appear, to be added to the potent chemicals such as β-propiolactone and aflatoxin.

Comparison of published data with ours shows that 9 of our 29 compounds have been reported. Deichmann *et al.* (5) indicated γ-valerolactone (XV) as negative; our test group also was negative. Dickens and Jones (9) and Van Duuren *et al.* (30) reported γ-butyrolactone (XII) as negative by subcutaneous test in rats and by skin painting in mice; we elicited only 1 sarcoma in 15 mice. The same authors also used α-angelicalactone with negative results, as we did. Van Duuren *et al.* (31) also had no neoplastic response with 2,2,4-trimethyl-3-hydroxy-3-pentenoic acid lactone (XVII), and we found no activity. With *cis*-9,10-epoxystearic acid (XXII), the results of Van Duuren *et al.* (28) were negative with skin painting and positive with subcutaneous injection (27); we obtained 6 sarcomas in 70 mice. Lauroyl peroxide in Van Duuren's experiments also was subcutaneously positive (29) and cutaneously negative (30, 31); we obtained no tumors in 15 mice. Walpole *et al.* (32) tested glycidyl laurate (XXIV) and stearate (XXVI) and obtained subcutaneous sarcomas in rats; these compounds also elicited

sarcomas in our mouse study. Glycidyl oleate (XXV) was stated to be negative by Weil *et al.* (33), but it did yield sarcomas in our series. We conclude that, in general, there is reasonable replication of results with these 9 compounds. This, of course, is strong evidence that at least some of the carcinogenic responses have real meaning.

Our data impressed us with the difficulty of interpreting marginal responses on essentially a single bioassay method of s.c. injection in mice. The mice were selected because with more potent carcinogens other indices of carcinogenic activity may have been elicited, such as increase in tumors of the breast, lung, or lymphomas. In contrast with our parallel study on crude extracts of a series of fungi (4), where such effects were obtained and yielded greater credence that indeed there were active agents, almost all of our responses in this investigation are limited to subcutaneous sarcomas. These appeared if multiple, long-term injections were used but were not related to the dose of the chemical and were so peculiarly distributed as to suggest microepidemics. Undoubtedly, there were, or were introduced, other factors of correction that defy identification at this stage.

We do not share the "disenchantment" with the subcutaneous test site, as discussed by Bischoff and Bryson (3). These strange and unanticipated findings may be a greater challenge than the addition of more weak carcinogens to the hundreds already known. What is clear is also stated in the thoughtful discussions by Bischoff and Bryson; the concept of carcinogenic activity should not be simplistic, and a single type of response in a single animal species is not adequate to designate the all-or-none implication of carcinogenicity or noncarcinogenicity. This is especially true of new, unknown, unrelated chemicals or mixtures, and may be less true when a well-typified group of related chemicals is being studied for chemical-biological correlates. Thus, single-type responses with the end point of tumors, whether these be subcutaneous sarcomas in rats, hepatomas in newborn male mice, or pulmonary tumors in strain A mice, are merely indications for further study in additional bioassay systems.

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MITOCHONDRIAL SWELLING AND UNCOUPLING ACTIVITY OF LONG-CHAIN FATTY ACIDS.

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In 1960 SCHOLEFIELD[1] reported the inhibition of phosphorylation in the cancer cells by fatty acids and in 1956 LEHNINGER[2] found uncoupling oxidative phosphorylation and swelling action[3] of fatty acids and of endogenous uncoupling factor such as unsaturated long-chain fatty acids, on mitochondria. In 1956 PRESSMAN and LARDY[4] reported that various fatty acids stimulated the DNP-stimulated latent ATPase activity of mitochondria. In spite of these findings the relationship among the oxidative phosphorylation, mitochondrial swelling and latent ATPase has remained unclarified. For the purpose to elucidate this point, the authors carried out a series of follow-up studies using various fatty acids. As the result, it has been found that there is an interesting relationship among these uncoupling oxidative phosphorylation, latent ATPase and swelling of mitochondria, and that the mitochondrial swelling induced by fatty acid is inhibited by various respiratory inhibitors especially azide and anaerobiosis.

MATERIALS AND METHODS

Mitochondria from rat liver and Ehrlich ascites tumor cells (6 days after transplantation) were prepared according to HOGEBOOM and SCHNEIDER's method[5]. These mitochondria were resuspended in 0.25 *M* sucrose solution (1 g tissue equivalent mitochondria of rat liver cells per 1 ml of sucrose and 5 g tissue equivalent mitochondria of Ehrlich ascites tumor cells per 1 ml of sucrose) as stock mitochondrial suspensions. Lauric, myristic, palmitic, behenic, elaidic, oleic, linoleic, linolenic and arachidic acids obtained from Tokyo Kasei Co. were purified and served as the reagents. ATP, ADP and antimycin A were obtained from Shigma Chem. Co., azide, KCN from Katayama Kagaku Co. and amytal from Yamanouchi Seiyaku Co. These reagents were all dissolved with 0.15 *M*

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KCl−0.02 *M* Tris-HCl buffer (pH 7.4) solution to keep certain ionic strength in order to maintain the mitochondrial function. Fatty acids were also diluted with the same solution in the form of Na-salt.

The mitochondrial swelling was measured by the method of LEHNINGER[5]. Stock mitochondria were washed 3 times with 0.25 *M* sucrose. Incubation mixture was composed of 4.75~4.4 ml of 0.25 *M* sucrose −0.02 *M* Tris −HCl buffer (pH 7.4) or 0.15 *M* KCl − 0.02 *M* Tris −HCl buffer (pH 7.4), 0.2−0.5 ml of 1 *mM* fatty acid (final concentration 0.04~0.1 *mM*) and 0.1−0.05 ml of stock mitochondria. The incubation was conducted at 37°C for 60 minutes. The incubation mixture was introduced rapidly to Beckman spectrophotometer and the extinction was measured at 520 mμ for the period of 60 minutes from the start, at the interval of 5 minutes. In this instance the concentration of fatty acids was adjusted to suit the purpose of each experiment.

Inhibitory effect on mitochondrial swelling induced by fatty acid was tested by respiratory inhibitors, such as 1 *mM* amytal, 10 γ / 5 ml antimycin A, 5 *mM* azide and 1 *mM* KCN, as the final concentration, and by the anaerobiosis.

The changes in the absorption at 520 mμ were observed to examine reversible contracton of the swollen mitochondria induced by fatty acid[6] with addition of 5 *mM* ATP, 5 *mM* Mg^{++} and 3 mg of bovine serum albumin (BSA) to the incubation mixture.

The uncoupling oxidative phosphorylation by fatty acid was studied by Warburg manometric method and by TAKAHASHI's method[7] for the determination of J10P fraction of mitochondria. The vessels contained 1.92 ml of stock mitochondrial suspension (1g tissue equivalent mitochondria suspended in 5 ml of 0.25 *M* sucrose solution), 0.20 ml of 0.4 *M* of sodium succinate and 0.3 ml of Krebs-Ringer phosphate solution in main chamber, 0.2 ml of 20% KOH in center well, and 0.28 ml of 0.4~1 *mM* sodium oleate in the side arm. The gas phase was air and incubation temperature was 38°C. For the estimation of the incorporation of ^{32}P into J10 P fraction, the incubation mixture contained 2 ml of 0.15 *M* KCl −0.02 *M* Tris buffer (pH 7.4), 2 ml of stock mitochondrial suspension, 0.6 ml of 1~4 *mM* of sodium oleate (in control system 0.6 ml of physiological saline solution) and 0.3 ml of Krebs-Ringer phosphate containing the labeled phosphate 10 μc. The medium was incubated for 30 minutes at 20°C in air environment. After the incubation, the reaction mixture was centrifuged at 0°C for 10 minutes at 14000×g, washed 3 times with 0.25 *M* sucrose solution to eliminate the contamination of absorbed ^{32}P. The acid soluble phosphate compounds in mitochondria were extracted with 3 ml of 5 per cent trichrolo-acetic acid for 30 minutes at 0°C and centrifuged for 10 minutes at 1700×g. One ml of the supernatant was used for the quantitative measurement and for counting radioactivity of phosphate. Another one ml of the supernatant was hydrolysed

with one ml of 1.5 N H_2SO_4 for 10 minutes at 100°C and the quantity and radioactivity of Δ10P were estimated by the TAKAHASHI's method[7].

On the other hand, oxygraphic method was employed to estimate the oxygen consumption and phosphorylation of rat liver mitochondria. The oxygraphy was constructed by one of the authors, K. UTSUMI, which is a slightly modified form of HAGIHARA's[8]. The incubation mixture consisted of 0.05 M sucrose, 0.02 M KCl, 0.02 M K-phosphate, and 0.1 mM EDTA (pH 7.5). Two ml of the incubation mixture was introduced to the sample cell of oxygraphy and then 0.2 ml of stock mitochondrial suspension was added to the incubation mixture (state I and II). After 1 minute 0.02 ml of 1 M sodium succinate was again added (state IV) and 1 minute later 0.02 ml of 10 mM of ADP was added (state III). After reversing to state IV, 0.04 ml of 4 mM of sodium salt of fatty acid (0.08 mM in final concentration) was added and after lapse of 1 minute 0.02 ml of 10 mM of ADP was again added. Then the effects of fatty acids on the oxygen consumption and oxidative phosphorylation were estimated by the ratio both before and after the treatment of fatty acids of respitation and oxidative phosphorylation in the presence of succinate.

The incorporation of ^{32}P into the acid soluble organic phosphate compound fraction of mitochondria was observed to prove the effect of fatty acid on the phosphorylation of mitochondria. The incubation mixture contained 3 ml of mitochondrial suspension (5 g tissue equivalent of mitochondria per 3 ml of 0.25 M sucrose solution), one ml of Krebs-Ringer phosphate containing 100 μc of ^{32}P, 1 ml of 0.2 M sodium succinate and 5 ml of 1 mM sodium oleate (oleate was replaced by the KCl-Tris solution in the control system). After incubation for 30 minutes at 25°C the acid soluble compounds were extracted with 5 per cent perchloric acid, neutralized with 1 N KOH and absorbed to Dowex 1 (× 4, 200 - 400 meshes) of formate type and eluted with formic acid and ammonium fromate according to the method of TERADA[9].

RESULTS

Effect of various fatty acids on the mitochondrial swelling of rat liver and Ehrlich ascites tumor cells: Generally, it is well known that fatty acids act as the uncoupler of oxidative phosphorylation and the damaging reagent of biological membrane structure. These facts suggest that the fatty acid acts as swelling-inducing reagent. As shown in Fig. 1 the swelling action of various fatty acids on the mitochondria of rat liver cells fluctuates according to the number of carbon chain in saturated fatty acid. The strongest activity is observed in carbon$_{14}$ myristate at the concentration of 0.1 mM. The swelling activity of C_{12} laurate and C_{16} palmitate are lower than that of myristate and the lowest are of C_{18} stearate and C_{22} behenate. On the other hand, in the un-

saturated fatty acids, the strength of their swelling action is in the descending order of linoleic, linolenic, richinoleic and oleic acids (Table 1). In this case, however, elaidic acid which is in the trans form of oleic acid shows the lower rate of activity than that of oleic acid. These differences appear at the period of 5 minutes incubation at 30°C, the rate of swelling is not much different among them at the period of 10 minutes. This means that there is a difference in the kinetics of the swelling action between trans- and cis- forms. On the whole the swelling action of these unsaturated fatty acids is stronger than that of saturated fatty acids (Fig. 1 and Table 1).

The kinetics of mitochondrial swelling induced by fatty acid is changed by the concentration of fatty acid and incubation mixture. As shown in Figs. 2 and 3 the degree and initial velocity of mitochondrial swelling are increased by the increment of oleic acid concentration and the swelling is inhibited by sucrose.

Table 1. Effect of unsaturation of C_{18} fatty acids (sodium salts) on the mitochondrial swelling. The test system is as in Fig. 1 and expessed the rate of swelling by fatty acid to spontaneous one.

Reagent	Unsaturation	Rate of swelling			
		After 5 minutes		After 10 minutes	
		0.1 mM	0.0025%	0.1 mM	0.0025%
Stearic	None	1.84	2.5	9.6	8.0
Oleic	9, cis	50.2	41.2	83.6	69.8
Elaidic	9, trans	10.3	8.5	60.0	49.2
Richinoleic	9,	56.1	40.6	75.7	63.8
Linoleic	9, 13, cis	92.1	76.3	93.4	77.6
Linolenic	9, 13, 17, cis	80.4	67.0	91.8	68.0

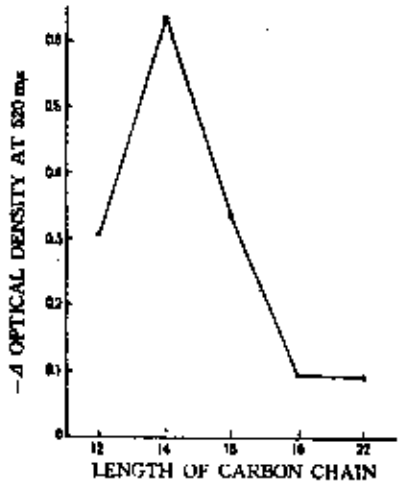

Fig. 1. The mitochondrial swelling action of saturated fatty acids (sodium salts) as function of carbon chain length. A medium of 5.0 ml of 0.15 *M* KCl-0.02 *M* Tris pH 7.4 was added to each tube, containing 0.1 m*M* of fatty acid. The changes of optical density were measured at 520 mμ at 37°C 20 minutes after the addition of washed rat liver mitochondria derived from 50 mg whole liver. The data are estimated with −Δ optical density at 520 mμ.

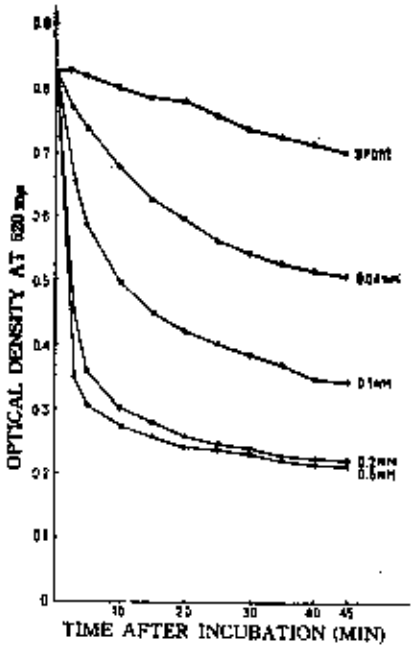

Fig. 2. Effect of various concentrations of sodium oleate on the swelling of mitochondria. The medium consisted of 5.0 ml of 0.15 *M* KCl-0.02 *M* Tris, pH 7.4. The amount of oleate shown was added, and optical absorbancy changes were measured at 520 mμ at 20°C after the addition of washed rat liver mitochondria derived from 100 mg whole liver.

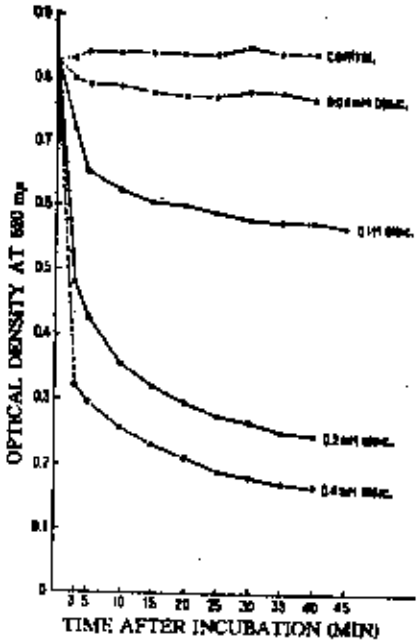

Fig. 3. Effect of various concentrations of sodium oleate on the swelling of mitochondria. The medium consisted of 5.0 ml of 0.25 *M* sucrose-0.02 *M* Tris, pH 7.4. The amount of oleate shown was added, and the reaction followed at 20°C as shown in Fig. 2

The minimum concentrations of oleic acid are found to be 0.04 *mM* in KCl solution but 0.1 *mM* in the sucrose solution as shown by LEHNINGER[8]. He[8] reported that the swollen mitochondria induced by various swelling agents can be recontracted by Mg^{++}, BSA and ATP. This recontraction of mitochondria is controlled by the presence or absence of contracting factor[10] on mitochondria. After the contracting factor is reduced by some swelling agents such as G-SH, cystein and Co A, recontraction does not occur by adding Mg^{++}, BSA and ATP. In the case of fatty acid induced swelling, however, the recontraction does occur and also the fatty acid induced swelling is inhibited by BSA and ATP. (Fig. 4)

In 1957 COOPER[11] reported the rate of swelling varies due to tissues which mitochondria are prepared from, and ARCUS[12,13] in 1960 stated that rat liver mitochondrial swelling induced by various agents was reduced in the process of

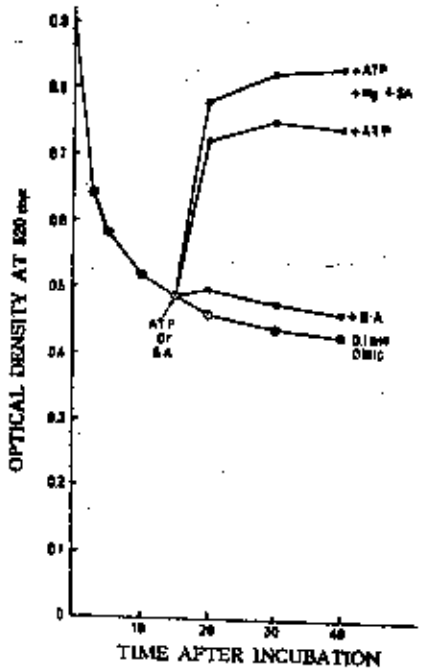

Fig. 4. Requirements for reversal of swelling induced by sodium oleate. The incubation mixture contained of 5.0 ml of 0.15 *M* KCl, 0.02 *M* Tris (pH 7.4), 0.1 *mM* sodium oleate and 100 mg tissue equivalent mitochondria. Additions were 5 *mM* ATP, 5 *mM* $MgCl_2$ and 3 mg per ml of bovine serum albumin. The reaction followed at 20°C.

carcinogenesis by DAB. In the present experiments the swelling action of oleic acid on various cell mitochondria was examined and the data were shown in Fig. 5. The swelling action is found to decrease in the order of the mitochondria from mouse liver, Ehrlich ascites tumor bearing mouse liver, Ehrlich ascites tumor cells and solid tumor of Ehrlich. The order of mitochondrial swelling seems to be parallel to there respiratory activity of each cell. In this respect, the inhibition of swelling of rat liver mitochondrial induced by various respiratory substrates has been examined using respiratory inhibitors such as amytal, azide, antimycin A, KCN, and anaerobiosis have been confirmed to inhibit the substrate inducing swelling (Table 2), which agrees with the reports of many investigators[14,15]. Oleic acid inducing swelling of rat liver mitochondria is also inhibited by anaerobiosis and by the respiratory inhibitors except amytal.

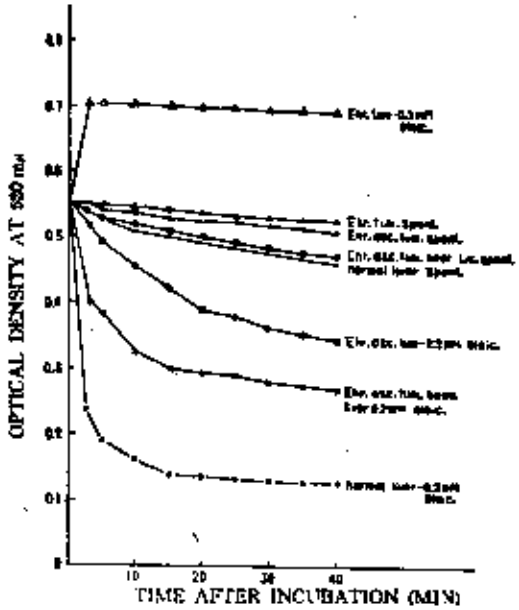

Fig. 5. Effect of sodium oleate (0.2 *mM*) on the mitochondrial swelling of mouse liver (50 mg), Ehrlich tumor bearing mouse liver (50 mg) and Ehrlich ascites tumor cells (250 mg) (solid and ascites). The details as in Fig. 2.

Table 2. Effects of respiratory inhibitors on the swelling of mitochondria by various substrates. The medium consisted of 0.15 *M* KCl-0.02 *M* Tris, pH 7.4 and 50 mg tissue equivalent rat liver mitochondria. Substrates and inhibitors present at zero time and incubated at 20°C 30 minutes. The data indicate the rate of absorbancy change to initial absorbancy at 520 mμ.

Substrate	Non	Amytal (1×10^{-3}M)	Ant. A (10γ/5ml)	Azide (5×10^{-3}M)	CN-(10^{-3}M)	Anaerob.
Non	89.0	79.5 (A)	89.0	92.0 (I)	92.0 (I)	
B-OH (3×10^{-3})*	73.1 (A)	95.2 (I)	79.5 (I)	88.5 (I)	88.2 (I)	
Succinate (α)	85.2 (A)	85.7 (I)	88.0 (I)	96.2 (I)	83.0	
Glutam. (α)	70.0 (A)	74.2 (I)	80.8 (I)	83.6 (I)	77.0 (I)	
α-KG* (α)	64.9 (A)		81.3 (I)			
Oleate(8×10^{-5}M)	26.1 (A)	16.0 (A)	28.2 (I)	41.2 (I)	27.6 (I)	29.5 (I)

* B-OH…β hydroxybutyrate α-KG…α-ketoglutarate A…Activation I…Inhibition

Effect of fatty acid on the oxidative phosphorylation of mitochondria: As has been reported previously[11], the uncoupling oxidative phosphorylation of the cell is brought about by fatty acids and it also occurs in mitochondria, i. e.

the respiratory activity of mitochondria is increased by the addition of 0.04 *mM* of sodium oleate but incorporation of ^{32}P into Δ10P fraction of mitochondria is inhibited severely as shown in Fig. 6 and Table 3. Mitochondrial respiration is released in the presence of oleic acid but after the lapse of 15 minutes at 38°C, the fall off is observed by the denaturation of mitochondria as shown in the cell level experiment[14]. This phenomenon is observable only in the intact mitochondria having the respiratory control[24].

By oxygraphic measurement of respiration and oxidative phosphorylation,

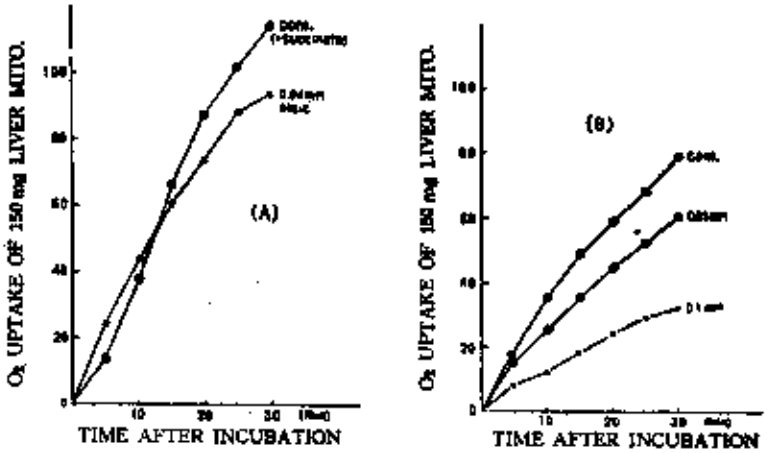

Fig. 6. Effect of sodium oleate on the respiration of rat liver mitochondria. Detail of incubation system as in the text.
(A) mitochondria was aged 3 hours at 0°C in 0.25 *M* sucrose solution.
(B) mitochondria was aged 3 hours at 0°C in 0.15 *M* KCl solution.

Table 3. Effect of sodium oleate on the ^{32}P incorporation into Δ10P fraction of rat liver mitochondria. Detail of incubation mixture as in the text.

	Ratio of RA of Pi	Ratio of RA of Δ 10P	Ratio of SA of Pi	Ratio of SA of 10P
Control	100	100	100	100
0.4 mM oleate	82.5	5.9	106	4.9
0.1 mM oleate	65.7	38.9	92	43.4

RA : relative activity SA : specific activity

the stimulated respiration under the existence of succinate and uncouping phosphorylation by adding of fatty acids are observed (Table 4 and Fig. 7). Parallel relationship can be observed between uncoupling oxidative phosphorylation and swelling action of mitochondria. (Fig. 1 and Fig. 7)

Table 4. Effect of various fatty acids (sodium salts) on the respiration and oxidative phosphorylation of rat liver mitochondria. Succinate level oxygen consumption and oxidative phosphorylation are expressed by mμ atom/min/100 mg tissue equivalent of mitochondria and P/O before and after the treatment of fatty acid (0.05 mM) by the method of oxygraphy.

Reagent (0.05mM)	Before treatment		After treatment	
	O_2 consumption (mμ atom)	P/O	O_2 consumption (mμ atom)	P/O
Lauric acid	30.0	1.54	45.0	1.09
Myristic acid	30.0	1.54	50.0	0.02
Palmitic acid	30.0	2.00	27.5	1.67
Stearic acid	30.0	1.67	15.0	1.54
Behenic acid	27.5	1.62	17.5	1.62
Oleic acid	30.0	1.67	50.0	0.01
Elaidic acid	30.0	1.62	32.5	1.54

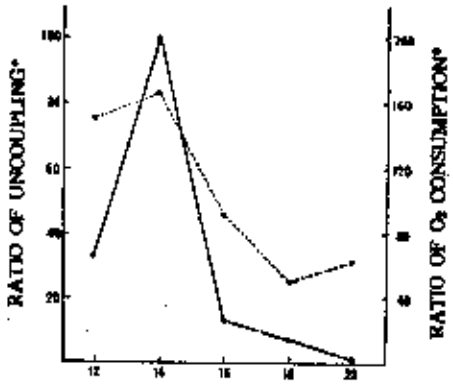

Fig. 7. Effect of various saturated fatty acids (sodium salts) on the respiration and phosphorylation of rat liver mitochondria. The medium contained as described in the text. (0.05 mM of sodium oleate and 100 mg tissue equivalent of mitochondria).

* Ratio of uncoupling ····· $\left(1 - \frac{\text{P/O of after treatment}}{\text{P/O of before treatment}}\right) \times 100$ ——·——

* Ratio of O_2 consumption····· $\frac{O_2 \text{ up take of after treatment}}{O_2 \text{ up take of before treatment}} \times 100$ ·····

The inhibition of ^{32}P incorporation into ⊥10P fraction of mitochondria by sodium oleate: From the data of uncoupling oxidative phosphorylation by fatty acid, the effect of oleic acid on the ^{32}P incorporation into acid soluble organic phosphate compounds is examined to reveal which fraction

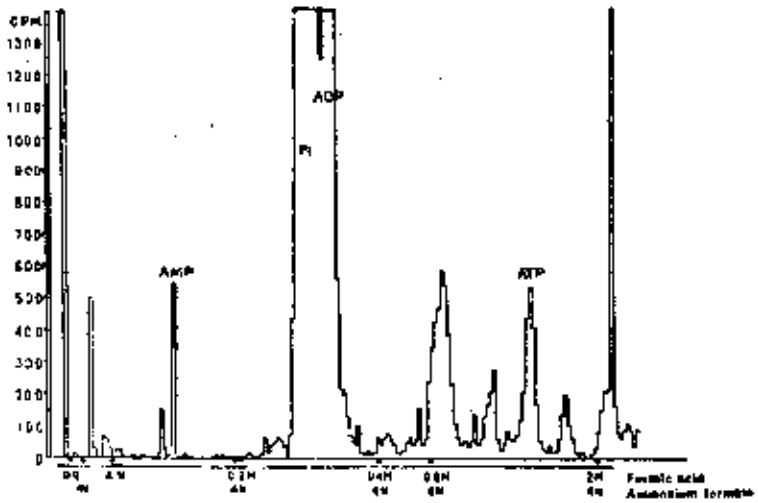

Fig. 8. Incorporation of ^{32}P into acid soluble phosphorous compounds of rat liver mitochondria (500 mg) after incubation 30 minutes at 25°C. The incubation mixture consisted with 3 ml of mitochondrial suspension, 1 ml of KRP solution (containing 100 μc of ^{32}P), 1.0 ml of 0.2 *M* sodium succinate and 5.0 ml of 0.15 *M* KCl-0.02*M* Tris buffer solution (pH 7.4).

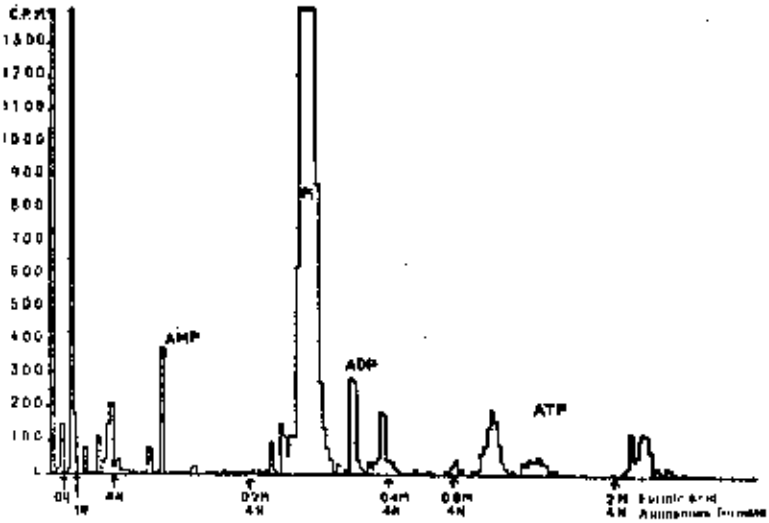

Fig. 9. The Effect of sodium iluate on the incorporation of ^{32}P into acid soluble phosphorous compounds of rat liver mitochondria. Incubation mixture containing 0.5 mM sodium iluate.

is suppressed by fatty acid in the mitochondria. As shown in Figs. 8 and 9 the typical inhibition of ^{32}P incorporation into ATP, ADP, GTP and UDPG are observed.

DISCUSSION

The mitochondria as the energy producing machine in the cell have been studied by many authors about the relationship between the structure and function. These experiments have drawn attention of many investigators on metabolic control mechanism related to the morphological changes of mitochondria. In this respect interesting results are reported[8,9,10-13] concerning the relation among the respiration, oxidative phosphorylation and swelling of mitochondria, especially noteworthy one is the recent report of PACKER[14] in which he found that the swelling-shrinkage of cancer cell is altered with the initial burst of respiration by adding of glucose (Crabtree effect). Namely, the metabolic control may be regulated by permeability of mitochondria according to swelling and shrinkage, which is caused by ADP, inorganic phosphate (Pi) and electron transport.

It is important to study the effect of fatty acid on the mitochondrial function on the following reasons: the fatty acid acts as uncoupler, swelling agent and stimulater of latent ATPase activity. In this experiment it has been clarified that the extent of uncouping action is parallel to that of mitochondrial swelling action of fatty acid as a function of the carbon chain length. This finding is similer to the stimulation of latent ATPase activity by various fatty acids as reported by PRUSSMAN and LARDY[4] and to the surface activity of each fatty acid. The above mentioned data suggest that the effect of fatty acid on the mitochondrial function could be decided by solubility of the acid into the lipoprotein of membrane structure and by damage to the functional structure of membrane. Thus the swelling of mitochondria could be induced. The swelling of mitochondria, therefore, means the structural change of membrane and will induce the loosened or uncoupled oxidative phosphorylation. It also means the stimulation of latent ATPase activity, which is a reversal process of the equation of phosphorylation proposed by LEHNINGER[15] and others. Even the swelling action is parallel to the uncoupling of mitochondria, an attention may be called on the difference in the incubation mixture in these cases: i.e. the swelling test is examined in the KCl solution using the mitochondria washed 4 times and uncoupling test is in the sucrose solution using the mitochondria after a single washing. When the swelling test is conducted in the sucrose solution with the mitochondria washed once, contraction occurs rather than swelling by adding of fatty acids. This fatty acid inducing contraction will be reported in a later paper. The medium used for testing the intensity of swelling and uncoupling, however, are

more effective than the sucrose medium. Moreover, the true P/O ratio would not be estimated by the method of oxygraphy because the difference between the added exogenous ADP and the endogenous ADP formed by stimulation of latent ATP-ase activity by fatty acid are in distinguishable. The experiments are being conducting to test whethere the stimulation of ATPase activity or uncoupling action by fatty acids cause the decrease of P/O ratio.

The mitochondria swollen by various swelling agents are recontracted reversibly by ATP, Mg^{++} and bovine serum albumin. This finding suggests that mitochondria contain the mechanoprotein as actomyosin in muscle. To support this idea OHNISHI[6] found in 1962 the actomyosin-, actin- and myosin-like proteins extracted from mitochondria and clarified that these proteins show cross reactions with actin and myosin prepared from skeletal muscle. In 1960 RACKER[27] also reported the coupling factor containing the latent ATPase activity. The mitochondrial membrane consists of structural protein, lipids and elementally particles[1]. From these findings it may be assumed that the mitochondrial structural protein contains the mechanoprotein may be orientated in some arrangement to an easily contractable state.

CORWIN[4] and others reported that respiratory inhibitors showed inhibitory action on the mitochondrial swelling induced by respiratory substrates, and the same results were likewise observed in this experiment. This means that the electron transport is one of the factors inducing the swelling of intact mitochondria. The inhibitory actions of azide and anaerobiosis on mitochondrial swelling induced by fatty acid suggest that the fatty acid may play a role as the respiratory substrate.

Cancer cell mitochondria showing low rate of swelling may be consisted of low saturated fatty acid as lipid component[28,29]. One of the physical properties of the lipoprotein is that it controls the rate of the swelling of mitochondria. These findings may be correlated to the regulation mechanism of cancer cell metabolism.

Recently a reversible uncoupling of oxidative phosphorylation has been demonstrated by SLATER[30,31], HOFSMANN[18] and PRESSMAN[4]: uncoupled of oxidative phosphorylation by oleic acid is reversed by the addition of serum albumin. These phenomena were also observed by our group (unpublished) but only at a low concentration of fatty acid. The mechanism of the reversible uncoupling reaction can be explained by the binding of serum albumin with fatty acid. In view of the reversible uncoupling concerned with the regulation of cell metabolism, unsaturated fatty acids are considered to be the regulator of cell metablism in natural system. Namely, the usual endogenous uncoupling factor or endogenous respiratory inhibitor contains some isoactane soluble fatty acids perphaps bounded to protein as cytoplasmic component as an inactive form. Then the regulation mechanism of cancer cell metabolism, differing from normal

one, may be aroused by the endogenous uncoupling factor and according to the data of specific lipid metabolism of cancer cells[9,10].

SUMMARY

The effect of various fatty acids on the swelling-contraction and oxidative phosphorylation of mitochondria from rat liver and Ehrlich ascites tumor cell have been studied and the results are as follows:

1. The swelling of rat liver mitochondria is induced by fatty acid. The extent of this uncoupling action is in the descending order of myristate, laurate, parlmitate, stearate and behenate in saturated fatty acid and linoleate, linolenceate, richinoleate and oleate in the unsaturated fatty acid. This swelling action is stronger with unsaturated fatty acids than that of saturated ones and cis form is stronger than trans form.

2. The uncoupling oxidative phosphorylation of rat liver mitochondria is also observed with these fatty acids and the activities are proportional to the degree of the swelling action.

3. The degree of swelling of rat liver mitochondria is proportional to the concentration of oleate and is inhibited by anaerobiosis and respiratory inhibitor except amytal.

4. The mitochondria swollen by fatty acid can be recontracted reversibly by ATP, Mg^{++} and bovine serum albumin.

5. The swelling action of sodium oleate is the strongest on mitochondria from rat liver, followed by those from the liver of Ehrlich ascites tumor bearing mouse, Ehrlich ascites tumor cells and solid Ehrlich tumor cells.

6. Sodium oleate inhibits the incorporation of ^{32}P into ATP, ADP, GTP and UDPG in mitochondria.

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[CANCER RESEARCH 32, 880–881, April 1972]

Letter to Editor

Replication of Low-Level Carcinogenic Activity Bioassays

Bioassay for carcinogenic activity of a number of epoxides, lactones, hydroperoxides, and peroxides was reported from the laboratory at New York University (2, 3). The compounds were tested by skin application on mice, s.c. injection in mice and rats, and feeding and intratracheal instillation in rats.

Another series of related compounds was more recently tested at Fels Research Institute by s.c. injection in mice (1). Included in this series were compounds such as stearic acid and γ-stearolactone which, on the basis of known structure-biological activity relationships and the known occurrence of some of these compounds as normal constituents of mammalian tissues, were not expected to be carcinogenic. Nevertheless, several of these compounds resulted in the induction of sarcomas at the injection site.

Because the results were difficult to evaluate, a meeting of investigators from the 2 laboratories was held in 1967. It was decided to repeat the bioassays of some of these compounds in 2 laboratories with the use of similar protocols and the same highly purified chemicals. We are communicating the results of this joint study.

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Five compounds were selected: stearic acid, methyl stearate, γ-stearolactone, *p*-nitroperbenzoic acid, and glycidyl stearate.

The same chemical samples and the same lot of tricaprylin as vehicle were used in both laboratories. The chemicals were given s.c. to mice once weekly for 26 weeks. The conditions of the experiments, as described in the reports (1, 3) were as identical as possible. The only difference was that the New York University experiments with ICR/Ha Swiss Millerton female mice, whereas the Fels experiments used CFW (Swiss Webster) female mice.

Table 1

Induction of sarcomas at injection site by s.c. injection in mice; results at 21 months

Compound	Laboratory	Dose (mg/0.1 ml tricaprylin) once weekly[a]	Total dose (mg)	No. of mice at start	No. of mice alive at 6 mo.[b]	Sarcomas at injection site	Other tumors
Stearic acid	A[c]	0.5	13	15	14	0	1
		0.05	1.3	15	15	0	3
	B	0.5	13	16	14	0	0
		0.05	1.3	16	13	0	0
Methyl stearate	A	5.0	130	15	15	2	1
		0.5	13	15	15	0	3
	B	5.0	130	16	16	2 (15)[d]	2
		0.5	13	16	16	0	0
γ-Stearolactone	A	1.0	26	15	14	0	0
		0.05	1.3	15	14	1	1
	B	1.0	26	16	16	1 (15)	3
		0.05	1.3	16	16	1 (18)	2
p-Nitroperbenzoic acid	A	0.05	1.3	15	15	2	0
		1.0	26	16	7	0	0
	B	0.05	1.3	16	16	1 (15)	4
Glycidyl stearate	A	0.1	2.6	15	14	1	3
		0.05	1.3	15	14	1	5
	B	0.1	2.6	16	16	1 (16)	3
		0.05	1.3	16	16	1 (16)	1
Tricaprylin	A	0.1 ml		15	15	0	0
	B	0.1 ml		16	16	0	1
No treatment	A			15	15	0	0
	B			32[e]	23	0	3

[a] Injected s.c., once weekly, for 26 weeks in inguinal area.
[b] From beginning of experiments. Median survival ranged from 15 to 21 months in test and control groups except for *p*-nitroperbenzoic acid at the higher dose (Group B only) where the median survival time was 5 months.
[c] A, New York University; B, Fels Research Institute.
[d] Nos. in parentheses, month when found.
[e] Some mice sacrificed for lung tumor observations to 6 months.

The results are summarized in Table 1. The replication of results in terms of s.c. sarcoma induction is remarkably similar in the 2 laboratories. Four of the 5 compounds induced sarcomas in less than 10% of the animals. One compound was negative in both laboratories.

Data on pulmonary and mammary tumors, as well as a few other tumors distant to the injection site, did not contribute further evidence of carcinogenic activity. Various neoplastic lesions were found in 20% of the group receiving glycidyl stearate and in 6 to 10% of all other groups.

Of the 4 compounds that induced sarcomas in mice, only 2 were expected to be active on the basis of known

structure-activity relationships (4): these are glycidyl stearate and *p*-nitroperbenzoic acid. The former is a monofunctional alkylating agent and the latter is a peracid. Both types of compounds have been shown in earlier studies to be carcinogenic (1, 2). The findings with methyl stearate and γ-stearolactone were surprising, since both of these compounds are relatively reactive and their conversion *in vivo* to reactive species is difficult to envision. We considered the possibility that "lipid-state" carcinogenesis was involved with some of these compounds, but they were lipid-soluble and gave clear solutions in tricaprylin at the concentrations used.

Low-level carcinogenic activity for the s.c. tissue of mice has been replicated in 2 laboratories for methyl stearate, γ-stearolactone, *p*-nitroperbenzoic acid, and glycidyl stearate, but interpretation of the data remains moot. It will therefore be desirable to test these compounds further, with other routes and additional species.

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Some Effects of High Lipid Diets on Intestinal Elimination
IV. Saturated Fatty Acids

By

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In a previous investigation made in this laboratory (1) some effects on intestinal elimination were noted when diets rich in glycerides of saturated fatty acids were fed to dogs. These effects included the following: (1) constipation, when diets containing large amounts of simple triglycerides of high molecular weight (tristearin and tripalmitin) were fed; (2) diarrhea, when small amounts of simple triglycerides of acids having twelve or less carbon atoms were fed. However, the addition to the diet of as much as 20 per cent trimyristin had no effect on intestinal elimination at all. Chemical analyses showed greater than normal amounts of lipids present in the feces when the glycerides fed melted above body temperature (tristearin, tripalmitin, trimyristin, trilaurin).

Several years ago, Bosworth, Bowditch and Giblin (2) noted that bottle-fed infants receiving diets too high in fat content were subject to vomiting and diarrhea. These investigators found increased quantities of calcium soaps present in the feces of such infants and attributed the gastro-intestinal behavior of the infants to the presence of these soaps. They also claimed that the fecal lipids would be further increased in such cases by neutral fats being occluded in the soaps.

Duell, Hallman and Reifman (3), who determined the rate of absorption of fatty acids in the rat found that butyric, caproic and caprylic acids fed as sodium salts were rapidly absorbed from the intestine, while capric acid (uncombined) was absorbed more slowly. Lauric acid became solidified and remained in the stomach so that it was absorbed very slowly. Lyman (4), feeding basic diets fortified with lard, ethyl palmitate, glyceryl tripalmitate, or palmitic acid to two dogs reported that the per cent of fatty acid (actually the ether extract) in the feces after ethyl palmitate or palmitic acid was much greater than after tripalmitin or lard feedings. He attributed the increase in ether extract to undigested ethyl palmitate in the first case, but the increase in palmitic acid when that lipid was fed could not be attributed to undigested material since no digestion was required. He, therefore, assumed that free fatty acids disturb the mechanism of digestion because they are irritating to the digestive tract, causing the food mass to be discharged more slowly from the stomach, but probably increasing peristalsis and the amount of feces.

Telfar (5), studying one normal infant and three with biliary atresia and obstructive jaundice, found that when bile is completely excluded from the gut, over 70 per cent of the dried feces are composed of fatty derivatives. Further, the non-absorption and persistence of excess fatty acids in the intestine are associated with modifications of the normal calcium and phosphorus excretion. Calcium is present in the feces in excess of normal, while phosphorus, released from calcium, is absorbed and eliminated in the urine. The extent of the variation is said to depend on the concentration of free fatty acids in the intestine.

While the preceding investigators believed that a relationship existed between dietary fat, fecal lipids and, in some cases, intestinal behavior, other investigators found no relation between ingested fats and fecal fat. Krakower (6), using eight fracture patients with good elimination habits as subjects, fed normal, low fat and special diets containing added corn oil (Mazola) or added coconut oil (Nucoa) for 4 day periods. Finding no correlation between the dietary fat and the total fat content of the feces or its composition as determined by the iodine number, he concluded that fecal lipids do not represent the residual food fat when moderate or larger amounts of fat are fed. Hill and Bloor (7), basing their conclusions on observations of four cats, decided that when moderate amounts of fat are fed, the fecal fat is independent of the diet, and that fecal fat ordinarily cannot be regarded as unabsorbed fat. They found the fecal lipids to consist principally of fatty acids and their salts (soaps) with smaller amounts of cholesterol and its derivatives and a little fat. They attributed the larger amounts of fat and fatty acids present in the feces after a high fat diet to unabsorbed food fat. Holmes and Kerr (8), using human subjects, also found the fecal fat to be different from that of the diet.

Because some of these investigators had attributed variations in either the behavior of the intestines or in the lipid content of the feces to the presence of unabsorbed fatty acids or calcium salts of fatty acids, it was decided to feed some of the fatty acids, the glycerides of which had been used in our previous experiment.

PROCEDURE

Four healthy male dogs*, the subjects in the previous experiment, were fed experimental diets. During this experiment, these dogs were kept indoors in sep-

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*Dog X, a thirty-pound, part-spaniel; Dog C, a mixture of hound and collie, weighing about twenty-three pounds; and two fox terriers R and W, weighing seventeen and thirteen pounds, respectively.

arate cages, from which they were removed for periods of exercise. All were fed once a day and at the same time. Originally all of the dogs were given a diet of canned Pard* Dog Food, but when this article disappeared from the local markets, Dog X was fed dried food in order to conserve the scarce tinned Pard for the dogs used for the quantitative determinations. The smaller dogs, C, W, and R, were each fed the contents of a one-pound can of Pard each day. When fatty acids were to be introduced into the diet, the contents of a can of Pard (454 gm.) were thoroughly mixed with the calculated amount of acid in a mortar. The dog was then allowed to eat directly from the mortar in order to avoid loss of material through a transfer to another dish.

The large dog X, as in the previous investigation, was used principally to test the palatability of the special diets. When this dog refused to eat the food or became ill after eating it, the concentration of added fatty acid was diminished to a point where such effects were not observed before the diet was fed to the remainder of the dogs. If the dogs did not eat all of the special diets, the food was removed from the cage after half an hour. The dogs were not fed again till the next regular feeding time, 24 hours later.

When the special diets were fed, information about the intestinal behavior of the animal was obtained, as in the previous investigation, by direct observation of the animal and by chemical examination of the feces. The fecal material was collected for examination and analysed as quickly as possible after defecation in order to avoid changes in water content.

The special diets were fed for three consecutive days; the second day's ration being identified by a marker, so that the time required for the food to pass through the intestines could be easily noted.

Chemical analyses, run on the sample after mixing and grinding in a mortar until homogeneous, included moisture, total lipids, lipids soluble in neutral ether and soap fat. Iodine and acid numbers were determined on the three lipid fractions.

Moisture determinations were run simultaneously with lipid determinations in a Kaye extraction apparatus with mercury seals (9). Lipids soluble in neutral ether were extracted for twelve hours with neutral isopropyl ether, soap fats were then removed by extracting the residues for twelve hours with decinormal acid isopropyl ether. Total lipids were obtained by extracting the original samples with decinormal acid isopropyl ether for twenty-four hours and the values compared with the sum of the lipids soluble in neutral ether and soap fat for the same fractions.

All of the lipid fractions were purified by shaking at frequent intervals with petroleum ether, during a period of eight hours. The fractions were then filtered, the solvent distilled off and the residue dried in a vacuum dessicator over concentrated sulfuric acid. Iodine numbers (Wij's Method) and acid numbers were determined for the purified lipid fractions.

*Sufficient canned Pard Dog Food to complete these studies was furnished us by the Research Division of Swift and Company.

DISCUSSION AND RESULTS

Because the dogs had eaten a diet of Pard containing 20 per cent added tristearin in the previous investigation, an attempt was made to feed a diet of Pard to which 20 per cent stearic acid had been added. However, the dogs refused to eat such a diet and not until the stearic content had been reduced to five per cent would they eat their food at all. On the second day, all of the dogs were badly constipated, listless and had elevated temperatures. They refused to eat all of their rations, the amount left being inversely proportional to the size of the dog (W, 200 g.; R, 100 g.; C, 78 g.). On the third day, the symptoms of the previous day were still present, and the dogs refused to eat at all. Twenty-four hours later, the animals seemed to have recovered and ate their normal Pard diet.

Table I contains the analytical data for the fecal material in which the marker for the second day of the stearic acid diet appeared. For the purpose of comparison, data for the tristearin diet, as well as the normal diet, have been included.

Inspection of Table I shows that the addition of as little as five per cent stearic acid to the usual Pard diet of the dogs caused a reduction in the moisture content of the feces. The total lipid content of the feces of two of the dogs was less than when Pard diet alone was fed. The per cent of neutral ether extract increased in every case, and the per cent of soap fats diminished, just as was the case when the triglyceride of stearic acid was added to the diet. However, the total amount of soap present after the feeding of a diet containing 5 per cent added stearic acid was greater than after the diet with 20 per cent added tristearin.

The analytical constants of the fractions furnish little information about the composition of the lipids in the fractions. However, it can be said that the soaps isolated from the feces after feeding stearic acid do not consist primarily of stearic acid salts, since the acid numbers indicate material of much higher molecular weight.

The acid numbers ranging from 43.7 to 50.4 indicate molecular weights from 1283 to 1113, respectively, while the molecular weight of stearic acid is only 284. It, therefore, seems probable that, when stearic acid is fed, a great deal of the so-called soap extract in the feces does not consist of soaps of fatty acids at all.

The iodine numbers indicate a greater degree of unsaturation in the soap fraction when stearic acid was fed, than when either the Pard alone, or Pard with tristearin was fed.

Since there was no increase in calcium soaps in the feces even when stearic acid itself was added to the Pard diet, it seems reasonable to conclude that constipation resulting from a diet rich in tristearin or stearic acid is not caused by the presence of unabsorbed calcium soaps.

TABLE I

Analytical Data From Feces of Three Dogs Fed Tristearin and Stearic Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Fat by Extraction	Per Cent Lipids: Soap Fat by Diff.	Iodine Numbers: Total Lipids	Iodine Numbers: Neutral Ether Extract	Iodine Numbers: Soap Fat	Acid Numbers: Total Lipids	Acid Numbers: Neutral Ether Extract	Acid Numbers: Soap Fat
Pard	C	69.9	20.3	2.9	18.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
5% Stearic Acid		64.1	9.1	6.2	3.3	3.1	26.4	23.2	31.2	20.1	12.5	45.7
20% Tristearin		40.8	50.5	46.8	4.1	3.7	5.2	1.9	17.3	16.0	185.1	54.2
Pard	R	73.9	19.5	9.1	7.6	10.2	5.1	6.4	25.1	48.6	38.9	54.2
5% Stearic Acid		65.2	18.5	10.1	5.0	5.1	20.3	18.4	42.6	23.6	15.4	45.0
20% Tristearin		46.2	27.6	21.6	3.6	5.8	7.7	3.5	19.0	40.6	37.8	74.5
Pard	W	71.8	17.4	5.0	13.1	12.4	12.9	18.2	12.0	56.2	29.1	43.4
5% Stearic Acid		69.5	27.6	16.4	10.5	11.2	16.4	12.8	53.4	18.3	14.7	50.4
20% Tristearin		54.7	17.6	59.4	0.9	8.2	5.6	5.5	35.6	46.5	37.1	118.5

Because the dogs had all been made ill by the addition of five per cent stearic acid to the Pard diet, the experiment was not duplicated with palmitic acid. However, Dog C was fed a diet of Pard to which ninety-three days all of the dogs ate a diet of Pard containing ten per cent additional lauric acid. During this period, the dogs were in good health and intestinal elimination was regular. The feces appeared normal in shape and consistency. It will be recalled that a Pard diet with 20 per cent added trilaurin in the previous

TABLE II

Analytical Data From Feces of Dog C Fed Tripalmitin and Palmitic Acid in Measured Quantities

Diet	Per Cent Lipids: Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Extract	Per Cent Lipids: Soap Fat Difference	Iodine Numbers: Total Lipids	Iodine Numbers: Neutral Ether	Iodine Numbers: Soap Extract	Acid Numbers: Total Lipids	Acid Numbers: Neutral Extract	Acid Numbers: Soap Extract
Pard	69.9	20.3	2.9	18.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
Pard+0.9% Palmitic	70.6	15.4	12.0	4.7	6.4	16.2	16.7	--	153.7	194.2	154.3
Pard + 20% Tripalmitin	63.5	36.2	42.7	9.8	14.2	5.3	7.4	8.4	96.6	83.8	201.2

tenths of a per cent palmitic acid was added. After this diet had been fed for three days, elimination was still normal. Analytical data for the feces included in Table II are not particularly informative.

The feeding of a meal of Pard to which lauric acid had been added was not difficult to accomplish. For investigation (1) caused diarrhea with feces only semi-solid in consistency.

Table III contains the analytical data for the fecal material eliminated after the feedings of Pard with

TABLE III

Analytical Data From Feces of Dogs Fed Trilaurin and Lauric Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract	Per Cent Lipids: Soap Fat by Extraction	Per Cent Lipids: Soap Fat by Difference	Iodine Number: Total Lipids	Iodine Number: Neutral Ether Extract	Iodine Number: Soap Fat	Acid Number: Total Lipids	Acid Number: Neutral Ether Extract	Acid Number: Soap Fat
Pard	C	69.8	20.3	2.9	18.0	17.4	8.0	27.2	5.6	52.8	49.4	59.9
10% Lauric		48.9	12.3	7.4	5.0	4.9	15.1	12.7	16.5	18.0	15.0	42.4
20% Trilaurin		81.2	*54.6	42.0	12.6	.	---	14.4	26.8	-	125.8	157.5
Pard	R	73.9	19.5	9.1	7.6	10.2	5.1	6.4	25.1	48.6	38.9	54.2
10% Lauric		68.8	14.6	8.0	6.5	6.2	11.9	11.2	15.4	18.4	17.4	42.9
20% Trilaurin		78.4	15.0	9.3	1.5	3.7	23.0	22.8	48.1	241.2	199.7	214.8
Pard	W	74.8	17.4	5.0	13.1	12.4	12.9	18.2	12.0	56.2	29.1	43.4
10% Lauric		72.0	13.6	7.4	6.1	6.2	13.9	12.4	17.1	18.7	19.4	46.3
20% Trilaurin		79.7	18.8	15.1	2.0	3.7	17.6	18.1	36.0	235.7	200.4	244.1

*Total Fat by Calculation.

lauric acid. Data from the trilaurin diet are added for ease in making comparisons.

Inspection of Table III reveals a slight reduction in moisture content in the case of the two terriers and a marked reduction for Dog C. This is in decided contrast to the increase in moisture content after the feeding of the triglyceride of lauric acid.

The agreement between similar lipid fractions obtained from the feces of all three dogs on the lauric acid diets should be noted. In all cases, the percentage of total lipids is less than when Pard alone was fed. The extremely low acid numbers of all the soap fractions show that lauric acid (or its salts) is not the principal component of the fecal fat when the acid is fed. An acid number of 42.4 indicates a molecular weight of 1323; lauric acid has a molecular weight of 200. This, again, is a contrast to the soap fat of the feces after feeding the glyceride of lauric acid. The higher acid numbers (187.3-214.5) indicate lower molecular weights (299-229) not too different from that of lauric acid (200). This is the best agreement between the molecular weight of the fatty acid fed and the molecular weight of the fatty acid in the material recovered from the feces of any lipid diet that we fed, excepting that of tripalmitin.

The dogs were reluctant to eat Pard containing added caprylic acid but finally consumed all of a mixture with five per cent of the acid. This diet, fed for three consecutive days, proved mildly laxative and caused semi-fluid stools.

No particular difficulty was encountered with a Pard diet containing five per cent added caproic acid for all of the dogs ate the diet with apparent relish. Following this diet, the stools were profuse, soft and pasty.

Three of the dogs could not be persuaded to eat Pard to which butyric acid had been added. Dog X, when denied other food, finally ate a mixture of Pard containing two per cent butyric acid (about 1 gm. in a pound of Pard). Following this feeding, the stools were pasty. Only part of one feeding of a mixture of Pard with four per cent butyric acid was eaten by Dog X. This feeding gave rise to pasty stools with much fluid. No chemical analysis of the stools was attempted.

The analytical data for the feces collected after diets with added caprylic and caproic acids are included in Table IV. The lipid content was much the same after the feeding of caprylic acid or its triglyceride in the concentrations used. Caproic acid, fed in the same concentration as caprylic acid, resulted in much larger quantities of fecal lipids than caprylic acid; in two cases more than the normal amount. Since the total lipids and not the soaps increased after the caproic acid feedings, it was at first suspected that the material might consist of unabsorbed caproic acid. However, the low acid numbers of the fractions indicate that free caproic acid was not present in the feces to any extent.

The low acid numbers of the soap fat present in the feces after the diets with caproic or caprylic acids indicate once more that calcium soaps are not formed in appreciable amounts, after large amounts of free fatty acids are fed.

The acid numbers of all of the soaps obtained from the feces of the three dogs after feedings with acids are in remarkable agreement. The only exception is the case of palmitic acid where less than one per cent was fed. If this acid is omitted from the list, the highest acid number of the soap fats is 50.4, obtained when stearic acid was fed to the smallest dog, the lowest value is 1.3, obtained when caprylic acid was fed to the largest dog. The average value for all dogs and all acids is 45.4. These acid numbers correspond

TABLE IV

Analytical Data from Feces of Dogs Fed Tricaprylin, Tricaproin, Caprylic Acid and Caproic Acid in Measured Quantities

Diet	Dog	Per Cent Moisture	Per Cent Lipids: Total Lipids	Per Cent Lipids: Neutral Ether Extract*	Per Cent Lipids: Soap Extract	Per Cent Lipids: Soap Fat by Difference	Acid Numbers: Total Lipids	Acid Numbers: Neutral Ether Extract	Acid Numbers: Soap Extract
Pard	C	69.8	20.1	2.9	18.0	17.4	52.8	49.4	59.9
5% Caprylic Acid		70.2	4.1	2.5	1.6	1.4	16.0	14.0	42.3
4% Tricaprylin		77.5	4.2	1.7**	1.1	. . .	88.6	93.7	95.4
5% Caproic Acid		70.1	25.0	18.5	6.7	6.7	17.3	14.0	42.5
2% Tricaproin		78.5	6.8	6.2	1.9	2.6	74.6	60.6	77.4
1% Tricaprin		76.1	5.1	3.6	1.0	1.5	153.4	141.9	106.6
Pard	R	71.9	19.5	9.3	7.6	10.2	48.6	38.9	54.2
5% Caprylic Acid		71.5	7.0	4.5	2.2	2.5	17.4	12.2	47.0
4% Tricaprylin		75.3	6.2**	5.7	1.0	.		91.2	108.8
5% Caproic Acid		72.8	22.4	16.9	5.4	5.5	15.2	12.0	45.0
2% Tricaproin		82.7	4.2	3.1	1.2	1.1	98.7	92.6	116.7
Pard	W	74.0	17.4	5.6	13.1	12.4	16.2	29.1	41.4
5% Caprylic Acid		73.2	2.9	2.5	0.8	0.4	18.2	20.1	49.1
4% Tricaprylin		76.8	7.8	6.4	0.7	1.4	108.3	91.4	128.5
5% Caproic Acid		71.9	11.4	9.5	3.0	3.9	14.0	11.4	47.1
2% Tricaproin		80.3	5.3**	4.7	0.8	--	.	69.6	123.4

* Mechanical Loss

** Total Fat by Calculation

to molecular weights of 1113, 1326 and 1235, respectively, a finding which strongly indicates that the materials did not contain any appreciable amount of fatty acid soaps. Samples of the so-called soap fats have, therefore, been preserved for further chemical analyses.

SUMMARY

1. Measured amounts of fatty acids mixed with a standard dog food (Pard) were fed to dogs to determine whether intestinal elimination would be affected by the added acid in the same manner as by the addition of the corresponding triglyceride.

2. Stearic, lauric, caprylic, and caproic acids were the acids mixed with the standard dog food and fed to all the dogs. One dog received feedings containing less than one per cent added palmitic acid; another ate a single feeding of Pard with added butyric acid.

3. Information concerning the intestinal behavior of the animals was obtained in two ways: (1) direct observation of the animal; and (2) chemical examination of the feces.

4. Chemical components of the feces for which analyses were made included moisture, total lipids, neutral ether extract and soap fat. Acid numbers were determined for all lipid fractions after all feedings; iodine numbers were determined after stearic and lauric acid feedings.

5. Constipation was produced by 5 per cent added stearic acid.

6. The neutral ether extract increased for every animal after the 5 per cent stearic acid feedings but the so-called soap fat was less than the normal value in every case.

7. The addition of 10 per cent lauric acid to the normal diet did not affect the normal rate of elimination of the dogs.

8. The feeding of diets containing caprylic, caproic or butyric acid produced laxative effects in all cases. The intensity of the action varied inversely with the number of carbon atoms just as in the case of the triglycerides of the corresponding acids. The moisture content of the feces did not vary greatly from the normal values when caprylic or caproic acid was added to the diet. No quantitative estimations were made after feeding butyric acid.

9. The acid numbers of all of the fatty acids from the soap extracts, with the exception of the one palmitic acid experiment, ranged from 42.3 to 50.4, with an average value of 45.4. These values correspond to molecular weights of 1326, 1113 and 1236, respectively.

CONCLUSIONS

The effect of a high fat diet on intestinal elimination depends at least partially on the component acids of the fats fed. Dogs fed pure saturated fatty acids behaved in approximately the same manner as when fed the corresponding pure triglycerides. Diarrhea resulted from the feeding of diets containing caprylic, caproic or butyric acids, or the glycerides of these acids. Constipation followed feedings of stearic acid or tristearin. Although trilaurin produced diarrhea, elimination was normal after feeding lauric acid.

These effects cannot be attributed to the presence in the feces of large amounts of free fatty acids or of soaps of the fatty acids, because the low acid numbers of the total lipid and neutral ether extracts exclude the possibility of large amounts of free acid. The low acid numbers of the soap fractions rule out the possibility of large amounts of soaps of fatty acids.

The low acid numbers of all of the fractions indicate that neither the fatty acid as fed nor its soaps are present in the feces to any extent. These low acid numbers can only be explained by assuming that small amounts of free fatty acids are mixed with large amounts of non-titratable material or by a very large molecule having an acid radicle.

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COMPARATIVE EFFECTS OF TALLOW AND COCOA BUTTER ON GROWTH AND SERUM LIPIDS IN CHICKS

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The effects of an animal and plant fat, having similar fatty acid patterns, on the growth rate and serum lipids of chicks were studied. Sixty male White Plymouth Rock chicks, housed in a battery, were fed a 24.6% protein corn-soy basal ration supplemented with 20% fat, using edible grade beef tallow or U. S. P. cocoa butter. After four weeks, the beef tallow fed chicks averaged 576 gm. gain while the cocoa butter fed chicks gained only 351 gm.

At four weeks, serum lipid analysis showed the following for beef tallow and cocoa butter, respectively (mean in mg. per 100 ml. ± S. D.): total lipids, 586 ± 27, 388 ± 13; neutral lipids, 378 ± 38, 183 ± 29; phospholipids, 210 ± 24, 202 ± 43; total cholesterol, 153 ± 3, 102 ± 3; triglycerides, 123 ± 14, 84 ± 12.

Serum lipid differences were not attributable to differences in feed consumption. Vapor phase chromatography of the serum fatty acids indicated no significant difference in serum polyunsaturated/saturated ratio. The mean oleic acid concentration with beef tallow was somewhat higher than with cocoa butter. The other fatty acids showed no significant differences. Poor absorption of cocoa butter would explain these results.

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The Energy Value of Fats and Fatty Acids for Chicks

I. METABOLIZABLE ENERGY

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IT HAS been shown by Donaldson *et al.* (1957) and Rand *et al.* (1958) that the chick can utilize high levels of fat as a source of energy. However, there are significant differences between fats in the amount of energy which is available to the chick (Carver *et al.*, 1955; Sunde, 1956; March and Biely, 1957). The magnitude of this difference was quantitatively demonstrated by Renner and Hill (1958) who determined the metabolizable energy value and apparent digestibility of a variety of fats ranging in iodine values from 44 to 169. These latter investigators also found that fatty acid mixtures derived from the hydrolysis of beef tallow, lard and soybean oil were much lower in metabolizable energy value than the respective intact triglycerides. At the same time they showed that both palmitic and stearic acids when fed individually were completely unutilized by the chick and attributed the poor utilization of the fatty acid mixtures to the free palmitic and stearic acids which they contained.

A number of investigators (Curtin and Raper, 1956; Combs *et al.*, 1958; Hathaway *et al.*, 1959) have reported that broiler chicks fed diets containing partially hydrolyzed fats and also fats composed wholly of free fatty acids showed a higher feed conversion than would be expected from the reported metabolizable energy values of some of these hydrolyzed fats and fatty acid mixtures. Therefore, the present investigation was undertaken to determine the utilizable energy and absorbability of the individual fatty acids in various fats and fatty acid mixtures when fed to chicks in a practical-type diet.

EXPERIMENTAL MATERIALS AND METHODS

Two experiments were conducted to determine the metabolizable energy value of a number of fats and fatty acid mixtures.

Experiment 1. The first experiment was designed to resemble closely the experimental conditions described by Hill and Anderson (1958). A semipurified basal diet similar to diet E-9 described by these investigators was used in this experiment.

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TABLE 1.—*Basal diet*

	Per 100 gram diet
Cerelose (glucose) (dry weight basis)	15
Soybean oil meal (50% protein)	35
Ground yellow corn	25.375
Isolated soybean protein[1]	4
Fish meal (60% protein)	5
Dehydrated alfalfa meal (17% protein)	2
Dried whey, delactosed	2
Distillers dried solubles	2.5
Vitamin mixture #3[2]	1.0
DL-Methionine	0.3
L-Lysine HCl	0.125
Dicalcium phosphate	2.2
Ground limestone	1.0
Mineral mixture #2[3]	3.5
Chromic oxide bread[4]	1.0

[1] Archer Daniel Midland C-1 Assay Protein

[2] This amount of vitamin mixture supplied: 400 mg. choline Cl (25% concentrate), 100 mg. vitamin B_{12} concentrate (1.3 mg./100 g.), 50 mg. stabilized vitamin A (10,000 IU/g.), 50 mg. dry vitamin E (220 units/g.), 2.0 mg. vitamin D_2 (200,000 ICU/g.), 4.0 mg. thiamine, 4.0 mg. niacin, 1.0 mg. Ca pantothenate, 0.4 mg. riboflavin, 0.3 mg. pyridoxine, 0.25 mg. menadione, 0.2 mg. folic acid, 0.03 mg. biotin, and 387.82 mg. glucose.

[3] The mineral mixture supplied: 0.8 g. iodized NaCl, 1.0 g. KCl, 1.5 g. $MgSO_4 \cdot XH_2O$, 0.04 g. $MnSO_4 \cdot H_2O$, 0.04 g. $FeSO_4 \cdot 7H_2O$, 0.005 g. $ZnSO_4 \cdot 7H_2O$, 0.002 g. $CuSO_4 \cdot 5H_2O$, 0.001 g. $CoCl_2 \cdot 6H_2O$ 0.0001 g. $NaMoO_4 \cdot 2H_2O$, and 0.1119 g. $CaCO_3$.

[4] Chromic oxide bread—30% Cr_2O_3 in wheat flour mixed to a thin dough with water, dried at 70°C., and ground to pass through a 40 mesh screen.

However, 2.5% corn oil was substituted for the hydrogenated vegetable fat, the thiamine level was doubled, and cobalt, zinc and molybdenum were added as trace elements. Each experimental diet was formulated by substituting the test fats, weight for weight, for glucose on a dry matter basis at 20% of the diet. Day-old Peterson × White Rock cockerels were fed the basal ration containing glucose for the first two weeks. They were then divided into 10 uniform lots of 10 chicks each and duplicate lots were fed each experimental diet containing the chromic oxide indicator. Excreta were collected during four 24-hour periods between the 21st and 26th days.

In the same experiment additional chicks were reared on the glucose basal diet for six weeks at which time duplicate lots of 10 chicks each were fed the various high fat experimental diets. After an eight-day adjustment period, excreta were collected over four 24-hour periods between the 50th and 55th days.

Experiment 2. This experiment was designed to measure the metabolizable energy value and apparent absorbability of a number of fats and fatty acid mixtures using a high protein diet made up of practical type ingredients. The fatty acids were prepared by hydrolysis of the respective fats. The basal diet is shown in Table 1. When 15% soybean oil is substituted for the glucose, this diet will provide a metabolizable energy Caloric to protein ratio of 60. It was calculated that the diet, containing the soybean oil, was adequate in all nutrients and would give a feed efficiency of 1.30 grams of feed per gram of gain at four weeks (Edwards and Young, 1959). A preliminary experiment established that this diet produced four-week growth of 615 grams and a feed efficiency of 1.35 grams of feed per gram of gain with Peterson × White Rock cockerels, which was in good agreement with the predicted value.

Arbor Acre White Rock cockerels were fed the glucose basal diet for the first week. They were then allotted to groups on an equal weight basis and were fed the experimental diets, which contained the various fats substituted for glucose on a weight for weight basis. Each diet was fed to duplicate lots of nine chicks each for eight weeks. Excreta collections were made on four consecutive 24-hour periods in the fourth and eighth weeks of the experiment.

At the end of each 24-hour collection period about 200 grams of excreta col-

lected from each pen were frozen immediately. The frozen excreta were dried in a forced air oven at 70°C., and equal amounts of dried feces from each of the four days' collections were ground together, mixed and stored in a sealed jar in the refrigerator until analyzed. In the second experiment an additional 50-gram sample of the frozen excreta from each pen was dried under vacuum at 37°C. These vacuum dried samples were later used as the source of fecal fatty acids which subsequently converted to methyl esters for gas-liquid partition chromatographic analyses.

Analyses. In order to calculate the metabolizable energy and absorbability of the test fats, the following analyses were made on both feed and excreta. Moisture was determined by drying a 4-gram sample to constant weight in a forced-draft oven at 105°C. Combustible energy was determined in a Parr adiabatic oxygen bomb calorimeter. Chromium was determined by the fusion method described by Brisson (1956). Fat was determined by weighing the petroleum ether extractible material obtained after saponification and acidulation of the feed or fecal samples, according to the following procedure.

A sample of air-dry material was finely ground in a micropulverizer or an Omni-Mixer. The percentage moisture was determined and a sample large enough to supply at least 100 mg. of fatty acid was used. To each gram of dried, ground material in a 125 ml. erlenmeyer flask was added 1.0 ml. of 50% aqueous KOH, 20 ml. of ethyl alcohol and a boiling chip. The mixture was refluxed for one hour. The flask was cooled and held in an ice bath while 20 ml. of distilled water were added, followed by sufficient concentrated HCl to give a pH of 1.0. The acidulated mixture was extracted six times with 30 ml. of redistilled petroleum ether (b.p. 35–50°C.). The combined petroleum ether extracts were evaporated on the steam bath to 30–40 ml. Granular anhydrous sodium sulfate was added to the flask and the mixture was allowed to stand at least 20–30 minutes.

The petroleum ether solution was filtered through fat-free or glass fiber filter paper into a tared erlenmeyer flask containing a boiling chip. The sodium sulfate was washed six times with petroleum ether. The filtrates were combined and the petroleum ether was evaporated under a stream of nitrogen on the steam bath. The flask containing the fatty acid was dried in a vacuum oven at 1 mm. pressure at 70°C. for one hour prior to weighing.

Calculations. The metabolizable energy value for the various fats was determined by the method described by Hill and Anderson (1958) where the fat was substituted weight for weight for glucose. The metabolizable energy value of glucose, the standard reference substance, was assumed to be 3.64 Cal. per gram on a dry matter basis (Anderson, Hill and Renner, 1958), and the metabolizable energy value of fats and fatty acids was calculated from the difference in metabolizable energy content of the reference diet and the fat-containing diet.[2] The absorbability value of each of the various

[2] Calories of Metabolizable Energy (ME) per Gram Test Material

$$= 3.64 - \frac{\text{ME for Cerelose Diet} - \text{ME for Test Diet}}{\text{Level of Substitution of Test Material}}$$

fats was determined by the following equation,[3] which corrects for the digestibility of the fat supplied in the ingredients of the basal diet.

$$\text{Absorbability of Test Fat} = \%\ \text{Utilization}^{4}\ \text{of Total Fat in Diet} \times \left(\frac{\%\ \text{Total Fat}}{\%\ \text{Test Fat}}\right) - \%\ \text{Utilization}^{4}\ \text{of the Fat in the Basal} \times \left(\frac{\%\ \text{Fat in Basal}}{\%\ \text{Test Fat}}\right)$$

In the second experiment the fatty acids isolated from the vacuum-dried excreta were converted to methyl esters by refluxing about 500 mg. of fatty acid in 50 ml. of 2% sulfuric acid absolute methanol solution. The methyl esters were extracted with petroleum ether and this solution was dried over anhydrous sodium sulfate. The solvent was removed under a stream of nitrogen in a warm water bath and the methyl esters were then dried under vacuum for 30 minutes. The methyl esters were subjected to gas-liquid partition chromatography at 185°C. in an Aerograph model A-90-C with a coiled 10 ft.×0.25 in. diameter copper tubing column packed with diglycolic acid polyethylene glycol polyester (Digipeg) supported on 60 to 80 mesh Celite #545, which had been acid washed and neutralized. Helium was used as the carrier gas at a flow rate of 37.2 ml. per minute (corrected to S.T.P.).

RESULTS AND DISCUSSION

The metabolizable energy values of the fats and fatty acids tested in the first experiment under dietary conditions similar to those described by Hill and Anderson (1958) are summarized in Table 2. These results show that the metabolizable energy value and absorbability of the corn oil in the four-week and eight-week old chicks are in good agreement with the values reported by Renner and Hill (1958). The value for edible beef tallow in the four-week old chicks was found to be a little higher than the 67% absorbability value reported by these workers. The metabolizable energy value and percentage absorbability of the hydrolyzed animal and vegetable fat, and the mixture of 90 parts hydrolyzed animal fat and 10 parts acidulated soybean oil soapstock were significantly higher than values reported by Renner and Hill (1958) for similar materials. Except for the metabolizable energy value of the latter, the various fats all showed a small increase in absorbability and metabolizable energy with the older chicks.

The description and analyses of the fats and fatty acid mixtures used in the second experiment with chicks fed the high protein practical type diet are shown in Table 3. The four- and eight-week growth and feed efficiency results of this experiment are summarized in Table 4. There were no appreciable differences in growth of the chicks on the various treatments

[3] The equation for determining the absorbability of the test fat was derived from the following relationship by solving for X.

Apparent Utilization of the Total Fat in Diet (footnote 4)

$$= \%\ \text{Utilization of the Fat in the Basal} \times \frac{\%\ \text{Fat in Basal Diet}}{\%\ \text{Total Fat in Diet}} + X\left(\frac{\%\ \text{Test Fat}}{\%\ \text{Total Fat}}\right)$$

where X = % Absorbability of the Test Fat.

[4] $\%\ \text{Utilization of Total Fat in the Diet} = 100 - 100\left(\frac{\%\ \text{Fat in Feces}}{\%\ \text{Fat in Feed}} \times \frac{\%\ Cr_2O_3\ \text{in Feed}}{\%\ Cr_2O_3\ \text{in Feces}}\right)$.

TABLE 3. *Analyses of the fats and fatty acids fed in experiment 2*

	Fatty acid		Unsaponifiable	Iodine value	Moisture
	Total	Free			
	%	%	%		%
Soybean oil	96.65	0	0.53	131	0.07
Corn oil	96.34	0.02	0.98	127	0.07
Lard	96.33	0.31	0.15	67	0.05
Beef tallow	95.90	0.33	0.12	45	0.07
Soybean oil fatty acids[1] (3–4 week experiment)	97.26	76.1	0.47	133	0.17
Soybean oil fatty acids[2] (7–8 week experiment)	99.06	92.8	0.51	135	0.23
Corn oil fatty acids[1] (3–4 week experiment)	97.9	87.5	1.06	130	0.18
Corn oil fatty acids[2] (7–8 week experiment)	99.2	93.2	0.89	127	0.26
Lard fatty acids[1] (3–4 week experiment)	97.6	94.7	0.10	69	0.80
Lard fatty acids[2] (7–8 week experiment)	99.3	95.4	0.15	67	0.19
Beef tallow fatty acids[2] (both 3–4 week and 7–8 week experiments)	99.1	96.4	0.10	45	0.18
Yellow grease	90.96	5.8	0.37	60	1.89
Hydrolyzed animal and vegetable fat	95.2	38.7	1.53	67	0.74

[1] Prepared by alcoholic potassium hydroxide saponification of the respective triglycerides for one hour followed by acidification and solvent extraction of the fatty acids.

[2] Hydrolyzed in a high pressure autoclave with 0.125% ZnO catalyst for 1.5 hours at 480–520°F. with a pressure of 600–700 psi. The hydrolyzed material was treated with 10% sulfuric acid to split the zinc soap. The fatty acids were decanted from the acid-water seat, washed with water until neutral, and vacuum dried.

compared to 52 for the feed grade tallow used by Renner and Hill (1958).

In this work it was also observed that corn oil fatty acids were as well utilized as corn oil itself.

These results show in general that the decrease in utilization of fats upon hydrolysis is greater for animal fats than for vegetable oils, but is not as great as that observed by Renner and Hill (1958) under their experimental conditions.

Gas chromatographic analyses of the fatty acids in the various fat and fatty acid mixtures and in the excreta of the four-week old chicks fed the various dietary treatments are summarized in Table 5. These values were used to calculate the absorbability of the individual fatty acids from each fat or fatty acid mixture. These results are summarized in Table 6. They show that the fatty acids in soybean oil, corn oil and lard are well utilized by the chick.

Renner and Hill (1958) have shown that palmitic and stearic acids when fed singly to chicks are not utilized. The re

TABLE 4.—*Utilization of various fats and fatty acids by the chicken*

Supplement	Growth and feed efficiency				Metabolizable energy and absorbability			
	4 weeks		8 weeks		3–4 weeks			7–8 weeks
	Average weight[1]	Feed/lb. gain[2]	Average weight[1]	Feed/lb. gain[2]	M.E.[3]	Utilization[4]	Absorbability	Absorbability
(15% of diet)	g.		g.		Cal./g.	%	%	%
Soybean oil	553	1.45	1,532	1.76	9.26	98	96	96
Corn oil	570	1.45	1,522	1.81	8.44	90	94	95
Lard	575	1.45	1,514	1.80	9.20	98	95	93
Beef tallow	548	1.68	1,464	1.90	6.56	70	72	76
Soybean oil fatty acids	565	1.43	1,510	1.79	8.26	88	95	93
Corn oil fatty acids	566	1.48	1,528	1.84	8.75	93	94	95
Lard fatty acids	538	1.49	1,469	1.83	8.40	89	94	83
Beef tallow fatty acids	535	1.59	1,448	1.96	5.71	61	69	67
Yellow grease	568	1.49	1,516	1.84	7.53	80	85	84
Hydrolyzed animal and vegetable fat	563	1.48	1,509	1.83	8.22	87	88	85
Cerelose	546	1.80	—	—	—	—	—	—

[1] Average of two groups of nine chicks each; all chicks were fed the basal diet containing 15% cerelose for the first week at which time they were allotted to groups on an equal weight basis.
[2] Feed efficiency figures based on the gain and food consumption from the end of the first week.
[3] Metabolizable energy.
[4] Metabolizable energy as percent of gross energy.

sults of this experiment show that when these saturated fatty acids are fed in a mixture of fatty acids such as the hydrolyzed beef tallow or lard they are utilized to a significant degree. Since this improvement in utilization is greater in the lard fatty acid mixture than in the beef tallow fatty acids it appears that the amount of unsaturated fatty acids present may influence the utilization of the saturated fatty acids. Whether this phenomenon can be attributed to any one specific unsaturated fatty acid cannot be ascertained from these data.

The hydrolysis of beef tallow caused a decrease in the absorbability of both palmitic and stearic acids, while in hydrolyzed lard only palmitic acid was decreased.

The hydrolysis of the vegetable oils

TABLE 5.—*Fatty acid composition of fats and fatty acids*

	As % of the fat used in diet					As % of fat recovered from excreta[1]				
	C_{16}	C_{18}	$C_{18}(1=)$	$C_{18}(2=)$	$C_{18}(3=)$	C_{16}	C_{18}	$C_{18}(1=)$	$C_{18}(2=)$	$C_{18}(3=)$
Soybean oil	11.5	4.3	27.3	49.7	6.9	22.9	9.2	27.8	33.6	3.3
Soybean oil fatty acids	12.3	4.5	30.0	46.7	6.4	20.0	13.1	34.9	23.1	2.7
Corn oil	12.0	2.7	30.1	54.7	—	25.9	7.6	27.3	33.3	1.2
Corn oil fatty acids	14.3	2.1	31.0	54.7	—	25.2	8.6	36.6	27.3	1.1
Lard	25.4	11.1	51.8	9.7	0.4	24.3	26.7	30.5	15.3	1.4
Lard fatty acids	26.2	12.1	50.2	9.6	1.1	46.1	24.7	19.3	6.5	0.9
Beef tallow	27.6	22.4	42.2	1.6	0.5	40.4	43.5	9.7	3.0	—
Beef tallow fatty acids	24.9	19.3	48.3	1.4	0.2	42.7	44.1	7.3	2.1	—
Yellow grease	25.1	16.2	46.9	8.7	0.4	30.0	33.7	27.1	7.2	0.5
Hydrolyzed animal and vegetable fat	25.1	17.0	42.0	11.7	0.2	33.8	36.3	19.1	9.2	0.3
Basal diet (no added fat)	22.9	4.7	23.6	40.5	4.1	27.8	13.5	27.9	23.2	2.1

[1] Average of duplicate determinations.

TABLE 6.—*Absorbability of fatty acids*

Source	Percent absorbability[1]				
	C_{16}	C_{18}	$C_{18}(1=)$	$C_{18}(2=)$	$C_{18}(3=)$
Soybean oil	90.8	93.4	95.6	96.5	97.5
Soybean oil fatty acids	92.0	84.6	93.3	97.4	97.7
Corn oil	87.0	89.4	95.3	96.3	—
Corn oil fatty acids	87.9	75.1	91.8	96.7	—
Lard	95.6	85.2	97.1	89.8	75.0
Lard fatty acids	87.4	85.9	98.4	98.9	96.0
Beef tallow	58.1	46.2	95.2	85.0	—
Beef tallow fatty acids	47.8	33.9	97.1	85.0	—
Hydrolyzed animal and vegetable fat	83.6	73.2	95.6	91.2	80.0
Yellow grease	82.4	68.6	92.1	87.6	71.2

[1] Corrected for absorbability of the fat in the basal ingredients and for endogenous fat as follows:

$$\%\ \text{Absorbability of Fatty Acid} = \%\ \text{Utilization of Total Amount of Fatty Acids in Diet}\left(\frac{\text{Total Amount of Fatty Acid}}{\text{Amount of Fatty Acid Added}}\right) - \%\ \text{Utilization of Fatty Acid in Basal Ingredients}\left(\frac{\text{Amount of Fatty Acid in Basal Ingredients}}{\text{Amount of Fatty Acid Added}}\right)$$

where % utilization of total fatty acid or fatty acid in basal ingredients

$$= 100 - 100\left(\frac{\%\ \text{Fat in Feces} \times \%\ \text{Fatty Acid in Fecal Fat}}{\%\ \text{Fat in Feed} \times \%\ \text{Fatty Acid in Feed Fat}} \times \frac{\%\ Cr_2O_3\ \text{in Feed}}{\%\ Cr_2O_3\ \text{in Feces}}\right)$$

caused a decrease in the absorbability of stearic acid. However, the decreased absorbability of stearic acid in the soybean and corn oil did not significantly affect total utilization of the fatty acids from these oils, as the stearic acid makes up only a small percentage of their fatty acids content.

The metabolizable energy values and absorbability values of soybean oil, corn oil, beef tallow and lard appear to be reliable measures for expressing the energy which can be utilized from these fats by the chick. Feed conversion figures from chicks fed diets containing these fats correlate with the absorbability values determined. In addition, values obtained in this laboratory, using a high protein diet made up of practical feed ingredients, agree well with the values reported by Renner and Hill (1958) using a semi-purified diet.

Although the metabolizable energy value of soybean oil fatty acids was in agreement with that reported by Renner Hill (1958), the absorbability values of not only this fatty acid mixture but also of the fatty acids derived from the hydrolysis of lard and beef tallow were significantly higher than values reported by those workers.

Similarly, the absorbability of the hydrolyzed animal and vegetable fat was found to be higher when determined under the conditions of these experiments than when determined under the conditions used by Renner and Hill (1958). The reason for this discrepancy is not apparent.

The beef tallow and beef tallow fatty acids used in the present experiments were from the same original material as those used by Renner and Hill (1958). However, the hydrolyzed animal and vegetable fat and the yellow grease used in these studies were from different sources and are therefore not directly comparable.

It is, however, interesting to note that the saturated fatty acids in mixtures of free fatty acids were significantly utilized

and that the degree of utilization increased as the amounts of unsaturated fatty acids in the mixtures were increased.

SUMMARY

The metabolizable energy values and apparent absorbability values of soybean oil, corn oil, beef tallow, lard and hydrolyzed animal and vegetable fat were determined with chicks fed a high protein diet made up of practical-type ingredients.

The metabolizable energy value and percentage absorbability of soybean oil, corn oil, lard and beef tallow agree with similar values determined by other investigators under other experimental conditions. However, the values for the free fatty acid mixtures, particularly those derived from beef tallow, lard and the hydrolyzed animal and vegetable fat, were significantly higher than values obtained by others for similar materials measured with chicks fed more purified diets.

Absorbability values of the individual fatty acids in the various fats and fatty acid mixtures were determined by gas phase chromatography of the methyl esters of fatty acids in the dietary and fecal fats. It was observed that the utilization of palmitic and stearic acids when fed in mixtures of free fatty acids was significantly improved as compared to the zero absorbability of these fatty acids when fed singly. The degree of utilization of these saturated fatty acids was greater in mixtures containing higher amounts of the unsaturated fatty acids.

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SC 2[illegible]

Induction of swelling of liver mitochondria by fatty acids of various chain length

Non-esterified fatty acids produce, in isolated mitochondria, manifold changes which may be summarized as follows: (a) stimulation of substrate oxidation in the absence of phosphate acceptor[1,2]; (b) activation of latent ATPase[3]; (c) inhibition of the ATP-P exchange reaction[4,5]; (d) uncoupling of oxidative phosphorylation[2,4]; and (e) induction of the so-called swelling of mitochondria[6,7]. Biochemical effects of fatty acids on mitochondria (points a to d) have been shown to be dependent upon the carbon chain length of the acids and upon the presence or absence of double bonds[3,8,9]. In contrast, little is known about the effects of chain length and of the presence of unsaturated bonds on the induction of mitochondrial swelling (point e), although a few data have been presented by AVI-DOR[7].

The present paper describes a study on the swelling effect exerted by fatty acids of the saturated series over the range of C_2 to C_{22} as well as by unsaturated acids and one hydroxy-monounsaturated acid.

Swelling of rat-liver mitochondria was measured photometrically at 520 mμ as described by LEHNINGER *et al.*[10].

Fig. 1 illustrates a typical experiment. It shows that the rate and the character of swelling was dependent on the chain length of the acids and on the presence of double bonds. The relationship between the swelling effect and the carbon chain

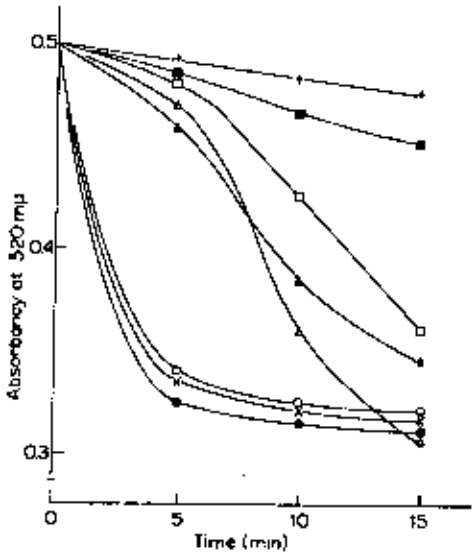

Fig. 1. Swelling of liver mitochondria induced by various fatty acids. Incubation mixture: 0.125 M KCl, 0.02 M Tris–HCl (pH 7.5), $1.33 \cdot 10^{-4}$ M sodium salts of fatty acids as indicated below, and rat-liver mitochondria containing about 5 mg protein; total volume, 7.5 ml; temperature, 20°. +—+, spontaneous swelling; ×—×, oleic acid; □—□, caproic acid (C_6), △—△, caprylic acid (C_8); ○—○, lauric acid (C_{12}), ●—●, myristic acid (C_{14}), ▲—▲, palmitic acid (C_{16}); ■—■, arachidic acid (C_{20}).

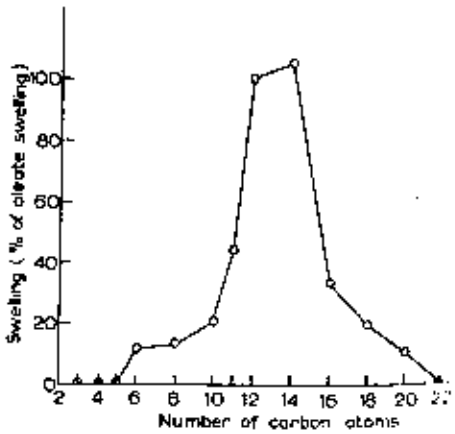

Fig. 2. Effect of saturated fatty acids on swelling of mitochondria from rat liver. Experimental conditions as in Fig. 1; incubation time, 5 min. Extent of swelling is expressed as per cent of the swelling produced by the same concentration of oleate during the same incubation time.

length of the saturated acids is seen in Fig. 2 where the swelling, during the first 5 min of incubation, is plotted against the number of carbon atoms. In order to facilitate comparison of results obtained from different experiments, the swelling produced by various acids is expressed as per cent of the swelling produced by the same concentration of oleate.

Fig. 2 shows that among the saturated acids the greatest swelling was produced by the acids of medium chain length (C_{12} and C_{14}). No swelling was obtained with fatty acids containing 5 carbon atoms or less, nor by behenic acid (C_{22}), even when these acids were used in a concentration ten times higher than that shown in Fig. 1.

A comparison of the swelling effect of saturated and unsaturated acids is shown in Table I. It is evident that *cis* unsaturated acids had a more pronounced swelling effect than saturated acids. Thus, oleic acid was a more potent swelling agent than stearic acid, and erucic acid was more active in this respect than behenic acid. On the other hand, the swelling effect of the *trans* isomer of oleic acid, namely elaidic acid, was almost the same as that of stearic acid. Additional unsaturation (linoleic acid) or hydroxylation (ricinoleic acid) or substitution of the triple bond for the double bond (stearolic acid) produced no further enhancement of the swelling effect as compared with oleic acid.

TABLE I

SWELLING EFFECT OF FATTY ACIDS

Experimental conditions as in Fig. 1.

Fatty acid		Swelling after 5 min incubation (as per cent of oleate swelling)
Name	Shorthand designation*	
Stearic acid	18:0	20
Oleic acid	*cis* 18:1[9]	100
Elaidic acid	*trans* 18:1[9]	17
Linoleic acid	*cis cis* 18:2[9,12]	98
Stearolic acid	18:1[9]	99
Ricinoleic acid	*cis* 18:1[9] hydroxy[12]	111
Behenic acid	22:0	0
Erucic acid	*cis* 22:1[13]	10

* The first number indicates chain length, the second indicates the number of double (:) or triple (:) bonds, and the index number the position of the unsaturated bonds.

The present results are in good agreement with those obtained by AVI-DOR[7], and reveal a striking similarity between the effect of various fatty acids on mitochondrial swelling and on the reactions of oxidative phosphorylation. Thus, PRESSMAN and LARDY[3] found that the most potent activators of mitochondrial ATPase were acids of chain length from 12 to 16 carbon atoms (compare Fig. 2 in ref. 3 with Fig. 2 in this paper), and that *cis* unsaturated acids were more active than the saturated acids. A similar dependence upon the number of carbon atoms has also been observed with respect to the inhibition of the ATP^{32}–P_i exchange reaction[8], the most inhibitory acids being those of medium chain length. Finally, a stronger uncoupling action of unsaturated acids on the oxidative phosphorylation, as determined by the decrease in P:O ratio, has been reported by BOREST *et al.*[9]. All these facts support the view[11]

that swelling of mitochondria is connected with the mechanism of coupling the electron transfer to the synthesis of ATP.

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