

VEGETABLE OILS, OLEIC AND
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CANCEROGENIC SUBSTANCES IN EDIBLE FATS AND OIL

Report IV: Experiments with Margarine,
Vegetable Shortening and Butter

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A topic that has frequently been discussed in articles, and which has also been the subject of numerous experiments, is the connection between the formation of cancer in the digestive organs and the fat included in food. For example, statistics have shown a correlation between food rich in fat and cancer, and in experiments with animals it has been demonstrated that high fat consumption favors the formation of cancer. Fats that were heated at a high temperature or for long periods manifested toxic effects. The cause of this is supposed to be a polymerization of the fats involving unsaturated fatty acids and an effect of autoxidized oils. The frequently expressed supposition that the heating of fats also produces a synthesis of aromatics was not, however, confirmed by our experiments. Rather, it was shown that a mere heating of salad oil to 320°C lowers the aromatic content. (Borneff and Fabian). Further results of this series of analyses concerned the detection of cancerogenic cancerogenic compounds in vegetable oils and their absence in bacon fat. Above all, this finding induced us to resume further investigations concerning fats, about which we report in the following.

Experiments with Margarine

Margarine is an emulsion of fat mixtures with skim milk or water and other additives in small concentrations. Quantitatively, the fat portion predominates.

In producing margarine, the fat and water phases are produced separately and then emulsified. The fat phase contains, besides the fat substances, the legally permissible fat soluble additives, such as dyes, vitamins, anti-oxidants and emulsifiers. The aqueous phase consists of acidified milk, water, aroma substances, common salt, egg yolk, preservatives and indicator substances. The warmed fat phase (35°C) and the cooled aqueous phase are emulsified together by means of stirring, beating or compressing. It is of course possible to produce

margarine from one fat, but in general several are used. The selection depends upon the raw material situation, the price and the quality of the margarine.

The composition of commercial margarine according to Kaufmann:

Fat (pure V.O. from December 15, 1965)	at least	80%
Water or milk (powder milk) and common salt	at most	20%
Emulsifiers, anti-spoilage and browning agents (lecithin, etc.)	varying e.g.	0.25-1%
Vitamin D		1000-5000 I.E./kg
Vitamin E, together with anti-oxidant		10-30 mg
Vitamin A		20,000-30,000 I.E./kg
Dyes:		
Carotene, together with provitamin A or orange 3 (bixin or annatto) + carotene		3000-5000 I.E./kg
Preservative (sorbic acid or sorbate)		0-1.2 g/kg
Sugar substances (syrup, glucose, milk sugar, etc.)		variable
Aromas (diacetyl, lactone, butyric acid ester, etc.)		variable
Starch flour as identifying agent		0.2-0.3%

The lack of essential fatty acids in margarines that existed earlier is balanced out today by the addition of oils rich in linolic acid. According to investigations made by Kaufmann (1955) ten types of commercial margarines revealed a 3% to 14% content of such unsaturated fatty acids; in 1964/65, Ristow reported 6-57% in 74 types of margarines, with the most frequent values between 10 and 20%. Even the objection to hardening of fat, that thereby glycerides of fatty acids form, the composition of which does not fully correspond to that of the natural ones, is no longer pertinent, for recently there has been success in steering the process in the desired direction. In judging margarine, besides the legal requirements and the nutrition physiological-hygienic requisites, the kitchen-technical suitability of the margarine is an important point of view, whereby appearance, taste, smell, structure, plasticity, homogeneity, color, fine decomposition of the water, browning upon heating, preservability, etc. play a role. The legal requirements concern above all the unobjectionable quality of the margarine (also of the initial products), fat content, water content, absence of forbidden preservatives, content of unsaponifiable fats, etc. The product also cannot be polluted bacteriologically.

The reports of the literature say that when produced correctly, the margarine is nutrition-physiologically irreproachable, and that there is no danger from foreign substances. (Coloring with the azo dye butter-yellow was forbidden as early as 1936, after Sasaki and Yoshida established its cancerogenic effect in animal experiments.)

We examined the following margarines (bought commercially on October 10, 1966):

Vegetable margarine, vitaminized	(= No. 1)
Pure vegetable margarine	(= No. 2)
Melted margarine for cooking	(= No. 3)
Table margarine	(= No. 4)

(Since, therefore, we examined a relatively small proportion of

the margarine brands available on the market, no brand names will be given, to avoid any discrimination, but rather only general characteristics will be indicated.)

Examples of fat mixtures (according to Franzen):

Table 1. Margarines composed chiefly of animal fats.

1. Quality		2. Quality		3. Quality	
Oleo margarine	40%	Oleo margarine	20%	Oleo margarine	20%
Premier juice	20%	Premier juice	30%	Premier juice	40%
Neutral lard	15%	Neutral lard	8%	Hard fat	10%
Sesame seed oil	10%	Hard fat	7%	Sesame seed oil	10%
Peanut oil	10%	Coconut oil	10%	Soybean oil	20%
Cottonseed oil	5%	Sesame seed oil	10%		
		Soybean oil	15%		

Table 2. Margarines composed chiefly of vegetable fats.

1. Quality		2. Quality		3. Quality	
Coconut oil	70%	Coconut oil	45%	Palmseed oil	50%
Neutral lard	10%	Palmseed oil	20%	Hard fat	20%
Sesame seed oil	10%	Hard fat	10%	Sesame seed oil	10%
Peanut oil	5%	Sesame seed oil	10%	Soybean oil	20%
Cottonseed oil	5%	Cottonseed oil	5%		
		Soybean oil	10%		

Analytical Method

The polycyclic, aromatic hydrocarbons were extracted with diethyl ether from the margarine after it had been saponified and, after a column chromatographic pre-separation, were chromatographed using basic Al_2O_3 on acetylated paper and analyzed (for a detailed description of the method, see Report II). The supplementary fluorescence-spectroscopic identification of the various aromatics can be found in Report III.

In order to obtain comparable values, we converted the aromatics content using the waterfree quantity of fat. Water determination was done according to the Karl-Fischer method.

Analysis of Coconut Fat

Coconut oil and coconut fat are obtained by means of compression or extraction from the copra, the dried rind of the coconut -- the fruit of the coconut palm (*Cocos nucifera* and *Cococ butyracea*), which is found in Southeast Asia, East and

West Africa.

The fresh coconut kernel contains 30-40% fat and about 50% water. It is dried either in the sun or in an oven. The dried copra is processed according to a two-stage method in oil mills. In order to obtain a good edible fat, the raw coconut fat must then be de-acidified, decolorized and deodorized. Characteristic for coconut fat is its high melting temperature, and the behavior while melting of this fat, which passes from the liquid to the solid condition within a very narrow temperature range. At the predominant temperature we used, the coconut oil represents a white or slightly yellow mass of tallow-like nature. 100% pure coconut edible oil is available on the market in various forms, e.g. as Palmin, Raw Coconut, Palmgold, etc.

We analyzed (purchased commercially in December, 1966):
Pure Coconut fat (= No. 5)

Analytical Method

The method corresponded to that used in the case of margarine.

Analysis of Butter

Butter is a water-in-oil emulsion, which is separated by means of "buttering" from the cream of cow's milk as an intimate mixture of milk fat and aqueous milk liquid. Additions of foreign fats are not allowed.

Butter therefore consists chiefly of fat, water, fat-free dry milk mass (egg white, enzymes, vitamins, milk sugar) and anorganic salts (the addition of common salt is allowed). The fat and water content is regulated by legal determinations. (Fat content at least 80%, common salt at most 3%, water at most 16% in the case of salted and 18% in the case of unsalted products.) The definition of the butter statute of June 2, 1951, differentiates between milk, cream and whey butter and that butter which is produced from sweet or acidified basic materials. Preparation of butter can be done according to various methods, which involve different kinds of butter formation, preparation of the cream, and different equipment. German commercial butter, German dairy butter and German national butter are differentiated qualitatively, the first being best and the last worst.

We analyzed (purchased commercially on October 10, 1966):
German commercial butter (= No. 6)
German dairy butter (= No. 7)

Analytical Method

The method corresponded to that used in the case of margarine.

Results

The results of the analyses are compiled in Table 3, into which, for purposes of comparison, some data concerning earlier findings are also entered.

Discussion of the Results

Table 3 shows that in bacon fat and butter, thus in animal fats, no cancerogenic aromatics can be found, while in margarines, coconut fat and vegetable oils, these compounds can be found in varying amounts. Thereby, the different cancerogenic activity of the individual aromatics must be observed (see Hoffmann and Wynder). Thus, for example, the total amount of cancerogenic aromatics in coconut fat is not significantly higher than in vegetable margarines, but the portion of 3,4-benzopyrene, which possesses the greatest activity, is four times as large as it is in the types of margarines analyzed. If first of all the total content of cancerogenic, polycyclic aromatics is considered, then the following succession is found: Margarine no. 1, coconut fat, margarine no. 3, margarine no. 2, margarine no. 4, vegetable oil, olive oil. If, however, we evaluate according to activity, then coconut fat stands first. Thus it results that the more expensive types of margarine, because of their coconut fat portion, are less favorable. Melted margarines, which contain no coconut fat, palmsced fat or palm oil, but rather 75-85% waltran or hardened peanut oil, do not, in contrast, have a high 3,4-benzopyrene content. We believe that the drying of the coconut fruits, which is done partly over an open fire, is responsible for an additional 3,4-benzopyrene formation or intake, i.e. one exceeding the gauge determined by nature. This intake is possible, for it is known that intense curing of copra yields a dark oil. Probably, therefore, the production of copra that is distributed over wide areas and countless islands produces inconsistent qualities. This problem is considered in the contribution of Grimmer. Beyond that, besides the publication of Howard et al., we know only of the publication of Malinia et al. After saponification of extracted and compressed sunflower seed oil and some margarine samples, the authors found 3,4-benzopyrene and other polycyclic hydrocarbons by means of fluorescence-spectroscopy. (Moreover, the results confirm part of our earlier results.) However, the details of the methods and data on the amounts of the aromatics found are not included in this congressional report.

In analyzing olive oil, earlier works probably failed because of detection methods that were too insensitive. With our operational method (see Report II) polycyclic aromatic hydrocarbons could be detected up to an amount of 0.1 μ g per kg fat, so that a negative finding can now be equated to a finding of "free of cancerogenic aromatics" fairly certainly.

The absence of polycyclic hydrocarbons in butter and bacon fat (see also Grimmer) can possibly be explained by the fact that aromatics introduced into the animal organism are hydroxylized and decomposed (see e.g. Butenandt and Dannenberg),

while the plants not only absorb the substances, but also synthesize them (Borneff, Gräf).

In the comparison between butter and margarine, the producers emphasize the fact that margarine is an independent food product and is not supposed to be an imitation of butter. The earth's population today is about 30% greater than it was before the last world war, but the use of butter has increased only slightly more than 10%, while that of margarine is more than double what it was. Thus margarine has indeed become a basic food product: consumption per capita in Germany amounted to 9.7 kg of a total edible fat consumption of 28.4 kg in 1965. However, the consumption of butter and margarine is different in different countries; still, the increase in margarine use (with the exception of Denmark) is clear in all cases (see Table 4).

Table 3.

A. Kanzerogene Aromaten $\mu\text{g}/\text{kg}^*$	Nr. 1		B. Margarine		Nr. 2		Nr. 3		Nr. 4		C. Cocos-fett		Nr. 5		D. Butter		Nr. 6		Nr. 7		E. Olivenöl		F. Schweinefett		G. Pflanz				
	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)	b)	a)				
1.2-Benzanthracen 1.	29,5	44,2	3,8	5,7	6,3	9,4	3,7	5,5	1,9	2,8	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,75	1,13	∅	∅	2,50			
3.4-Benzfluoranthen 2.	14,5	21,7	3,1	4,6	3,7	5,5	2,6	3,9	1,4	2,1	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,82	1,23	∅	∅	4,05			
3.4-Benzpyren 3.	2,7	4,0	5,5	8,2	3,0	4,5	2,2	3,3	12,4	18,6	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,62	0,93	∅	∅	0,35			
Indenopyren 4.	5,5	8,2	1,0	1,5	1,2	1,8	1,6	2,4	0,9	1,3	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,9	1,35	∅	∅	1,65			
10.11-Benzfluoranthen 5.	10,5	15,7	2,3	3,4	5,7	8,5	2,4	3,6	4,4	6,6	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,8	1,20	∅	∅	3,75			
Nichtkanzerogene Aromaten 6.																													
1.12-Benzperylene 7.	6,8	10,2	0,5	0,7	1,7	2,5	0,7	1,0	1,1	1,6	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,67	1,00	∅	∅	1,25		
11.12-Benzfluoranthen 8.	5,3	7,9	0,8	1,2	1,2	1,8	1,4	2,1	3,3	4,9	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	0,55	0,82	∅	∅	1,35		
Fluoranthen + Pyren 9.	55,5	83,3	67,9	101,8	12,1	18,1	4,3	6,4	63,0	94,5	1,0	1,5	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	12,75	19,13	11,5	17,25	16,30		
Menge an Kanzerogenen 10.	62,7	93,9	15,7	23,5	19,9	29,8	12,5	18,7	21,0	31,5	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	3,89	5,84	∅	∅	12,30	
Gesamtmenge 11.	130,3	195,4	84,9	127,3	34,9	52,3	18,9	28,3	88,4	132,6	1,0	1,5	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	17,86	26,79	11,5	17,25	31,20
Wassergehalt % 12.	16		18		0,07		14,2		0,3		13,8		14																

* in relation to waterfree substances

a) = values found

b) = corrected values (factor 1.5)

Key:

A. = cancerogenic aromatics B. = margarine C. = coconut fat
D. = butter E. = olive oil F. = Bacon fat G. = vegetable oil

1. = 1,2-benzanthracene 2. = 3,4-benzofluoranthene

3. = 3,4-benzopyrene 4. = indenopyrene

5. = 10,11-benzofluoranthene

6. = Non-cancerogenic aromatics

7. = 1,12-benzoperylene

8. = 11,12-benzofluoranthene

9. = fluoranthene + pyrene

10. = Amount of carcinogens

11. = Total amount

12. = water content in %

Since the analyses have shown that man takes in carcinogens along with vegetable oils and margarine, the question of possible danger is raised. Without doubt, this problem can only be judged from the standpoint of "syncarcinogenesis". In this

sense it is important that, along with cigarette smoking and the air of large cities, food also -- even the so-called natural food -- introduces carcinogens into the human organism (smoked fish and meat products, drinking water, vegetables, coffee, vegetable fat). Of course, no one of these foods can work as a sole cause of cancer, but in sum, they yield not innocuous amounts.

At this time, we estimate, according to the various data given in the literature, that the yearly dose of carcinogens with smoked food products (about 5 kg) is 0.05 mg, with drinking water in the form of appropriately processed surface water (about 1 m³) 0.025 mg, with vegetable foods (about 200 kg) 1-10 mg, and with margarine and vegetable oils (about 20 kg) 1 mg. In calculating the dose in margarine, we proceeded from the average values for Germany (Table 4) for 1965, thus the total yearly dose of carcinogens from sources recognized as unobjectionable is 1-10 mg with a 3,4-benzopyrene portion of 0.1-1 mg.

Table 4. Butter and Margarine use per capita (in kg/year) according to Diemair and Koch (1960) and the Commonwealth Economic Committee: Intelligence Bulletin, June 1967.

	Margarine		Butter	
	1938	1965	1938	1965
Norway	18.53	21.2	5.66	4.1
Denmark	21.47	18.3	8.29	10.0
Netherlands	7.25	19.6	5.44	4.3
Sweden	9.29	16.1	11.32	8.7
Federal Republic of Germany	5.98	9.7	8.60	8.4
Belgium	7.25	13.1	7.88	7.9
Finland	3.80	4.5	9.83	17.7
Great Britain	3.94	5.4	11.23	8.8
U.S.A.	1.31	4.5	7.43	2.9
Ireland	1.59	4.1	14.95	16.7
France	0.82	3.0	5.21	8.9
Switzerland	0.95	1.8	7.16	6.7
Italy	0.23	0.7	1.18	1.6

In evaluating this amount it should be said that with 0.5 mg 3,4-benzopyrene tumors could be caused on nice skin in 100% of the animals used after 3-5 months (Bryan and Shimkin). Smaller doses are by no means completely ineffective, only the tumoral capacity sinks in the face of increasing time of latency. Of course, the mouse skin is not entirely comparable to the human intestinal tract, but nonetheless the demand for elimination or at least limitation of the carcinogens seems sensible and justified.

In Germany, appropriate measures were undertaken with the legal prohibition of a series of foreign substances, whereby the stand was taken that cancerogenically acting compounds should in no case purposely be added to foods. This basic stand, the elimination of carcinogens within the frame of available technical possibilities, should also be valid in all other areas. Preventive medicine must demand corresponding precautionary measures, even if a final judgment concerning danger due to carcinogens in food cannot yet be made.

In this light, we consider it desirable to conduct further analyses of the aromatics in vegetable fats, and to select properly the raw materials and control the production process in order to reduce the polycyclic, aromatic carcinogens. According to Grimmer and Biernoth and Rost (see also following article), the raw material components of vegetable margarine have different aromatic contents; further, the authors could demonstrate that by means of proper processing, a decisive decrease in the hydrocarbon content can be effected. Such refining measures are not unusual in themselves, for it has already been seen several times that a plant "nature product" by no means possesses optimal properties in a nutrition-physiological respect. Turnip oil, for example, obtained by pressing and extraction, contains smaller amounts of sulfur compounds, which must be removed by refining, especially deodorizing. Cottonseed oil must be freed of gossypol and its esters, as well as of cyclic fatty acids (9,10-cyclopropene-stearic acid = stercylic acid). György, Goldblatt and Ganzin observed that red palm oil leads to cirrhotic alterations of the liver in animal experiments. Knowledge gained in the last few years has further revealed that toxic properties (curare, strophantin, aconitin, mushroom poisons) are not always recognizable from temporarily appearing symptoms, i.e. there are toxic substances in plants that can lead to intense phenomena only after quite a long period of latency. Besides the named substances, however, cancer-producing substances also naturally occur (senecio-alkaloids, cycasin, mycotoxins, monocrotalin, carrageenin, safrol, tannin, citrus oils), so that recognition of the chronically toxic dangers of nature products is of great practical significance (see e.g. Druckrey). In 1962, Kaufmann pointed out that for these and several other reasons, the plant world by no means offers better food products than the animal realm. We, however, are of the opinion that harm can be avoided by careful selection. Above all, we would not like to make the mistake of concluding a superiority of animal fats from the presence of 3,4-benzopyrene in margarine, especially since it can be expected that the margarine treatment recommended by Biernoth and Rost, with A-carbon and vapor deodorization will eliminate in practice harmful concentrations of polycyclic aromatics. It is not the task of our analyses to establish value judgments concerning different foods; they serve exclusively the purpose of base information for later prospective epidemiological ascertainment, which alone can decide upon the dangerousness or harmlessness of the traces upon long-term intake.

Summary

Analyses of various types of margarine, vegetable shortening and butter confirm the fact established in earlier research, that vegetable fats and oils, in contrast to animal products, contain cancer-producing, aromatic hydrocarbons. The aromatic content of the margarine types is determined from the raw products. The amount of cancerogenic aromatics in the margarines analyzed, produced without active-carbon treatment, and in coconut oil lay between 20-100 $\mu\text{g}/\text{kg}$, and the 3,4-benzopyrene portion amounted to 3-18 $\mu\text{g}/\text{kg}$. These compounds could not be detected in butter.

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Lipid Composition of Vegetable Oils

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I. Introduction

About 400 different botanic species are known to us today from whose seeds or fruits (mostly the former) oils can be obtained (or fats, when their melting point is above the average temperature of the original area); notwithstanding the similarities, very close at times, between oils of different origin, it is practically impossible to find two of them identical. Each has some distinctive chemical characteristic, infrequently physical, sometimes qualitative, more frequently quantitative.

A vegetable species can be called "oleaginous" only when its seeds or fruits can yield at least 15-20% oil; actually, any portion of a vegetable, if not excessively lignified, contains lipids, but only the ripe seeds (and, but less so, the fruits), contain a high percentage, accumulated as reserve material, which may be easily mobilized and metabolized to meet the demand for energy during germination.

From the origin of organic chemistry it was recognized that oils are complex mixtures of numerous products that are structurally related; this fact, in addition to the experimental difficulty in resolving such mixtures, is undoubtedly the reason why the chemistry of fatty materials has been for decades mainly qualitative and based on average "values." The extraordinary current advances in our knowledge of fats, in all fields involving them, are exclusively due to the possi-

bility of resolving in a precise manner the mixtures of similar compounds of an oil by means of the various chromatographic techniques on paper, thin-layer (TLC), and gas-liquid chromatography (GLC). Only after these techniques had allowed the identification and separation in pure form of compounds of closely related structures, such as fatty acids, could investigations be profitably carried out by means of spectroscopic methods: ultraviolet, infrared, nuclear magnetic resonance, and mass spectroscopy.

Today, research on the components of most different lipid types, vegetable oils included, is in full development; we may expect from it the solution of many problems in the medical-biological field, and also improvements in industrial applications. This broad subject is constantly progressing and cannot be summarized in a short review; several aspects will not be discussed in detail here, and the literature quoted will be confined mainly to the last decade.

II. Glycerides

A. FATTY ACIDS

Only a relatively small amount of fatty acids occurs in the free form in plants; however, in combined form, fatty acids are widely distributed in the vegetable kingdom and constitute the basic unit of many lipid molecules.

Fatty acids present in free or bound forms in vegetable oils usually have straight chains, an even number of carbon atoms, and are monocarboxylic; odd-numbered acids occur rather rarely and always in trace amounts. Branched-chain fatty acids have not yet been found in vegetable oils, but unusual fatty acids having acetylenic, cyclopropenic, hydroxy groups or conjugated double bonds are sometimes important constituents of seed oils.

1. Analytical Methods and Structure Analysis

Traditional chemical methods are currently used in the characterization of fatty acids: neutralization values permit the calculation of the mean molecular weight and a series of values (for instance, iodine, thiocyanogen, and hydrogenation values) for determining the degree of unsaturation. A complete analysis of fatty acid mixtures would nevertheless require the qualitative and quantitative detection of all the fatty acids (Fontell *et al.*, 1960). Such methods as crystallization of salts and bromine adduct formation have been used to some extent. Nowadays, less conventional methods have found wide

application; these include gas-liquid chromatography (GLC), column and paper chromatography, thin-layer chromatography (TLC), and countercurrent distribution (Butterfield *et al.*, 1964; Dutton, 1955; Holley, 1956; E. P. Jones *et al.*, 1965; Scholfield *et al.*, 1961c, 1963a,b, 1966; Sreenivasan *et al.*, 1963). GLC is the most versatile and widely used method for analysis of fatty acid mixtures; the fractionation of the different constituents is governed by several factors (Harris and Habgood, 1966; Horning *et al.*, 1964a; Lovelock, 1952), but, when proper conditions are chosen, an enormous range of resolving power is attained.

The separated components of a mixture can be detected in several ways (Burchfield and Storrs, 1962; Horning *et al.*, 1964b; Lovelock, 1952, 1958a,b; Karmen and Bowman, 1959; Messner *et al.*, 1959; Stuve, 1961); the data, proportional to the amount of the eluted material, can be recorded.

Retention times are reproducible for given columns and operating conditions; their logarithms, within a homologous series, bear a linear relationship to the chain length (Ackmaw and Burgher, 1963; Farquhar *et al.*, 1959; Haken and Souter, 1966; James, 1959; T. M. Smith and White, 1966; Woodford and Van Gent, 1960) so that fatty acids, usually analyzed as methyl esters (Burchfield and Storrs, 1962; Horning *et al.*, 1964b; Woidich, 1966), can be readily recognized by comparison to reference compounds or by plotting retention data for known compounds (Litchfield *et al.*, 1963; Miwa *et al.*, 1960). Routine analytical procedures, which include the use of several stationary phases, have been fully described for saturated and unsaturated acids (Burchfield and Storrs, 1962; Horning *et al.*, 1964b; R. B. Jackson, 1966a,b). Less common acids are equally well analyzed: hydroxy acids as trimethylsilyl ethers (Kitagawa *et al.*, 1962; O'Brien and Rouser, 1964; Wood *et al.*, 1965); polyhydroxystearates as trifluoroacetates (Wood *et al.*, 1966), epoxy acids (L. T. Black and Eisenhauer, 1963; Herb *et al.*, 1964; Morris *et al.*, 1961), olefinic and acetylenic acids (T. M. Smith and White, 1966), cyclopropenoid (Hammonds and Shone, 1966) and cyclopentanoic acids (Zeman and Pokorny, 1963). Isomers of straight-chain fatty acids can also be separated and analyzed by high resolution Golay columns (Juvet, 1964; Litchfield *et al.*, 1963; Mallard and Craig, 1966; Wood *et al.*, 1965); combinations of GLC with TLC, column chromatography, and countercurrent distribution are also helpful in analyzing complex mixtures of isomers (Iverson *et al.*, 1965; Mallard and Craig, 1966; Wieske, 1966; Wood *et al.*, 1965). Acids present in oils as artifacts have also been detected (L. T. Black and Eisenhauer, 1963). Paper

chromatography is now widely replaced by TLC, which is more versatile and is a faster operation. The reversed-phase procedure, employing as stationary phases hydrocarbons (Buchanam, 1959; Kaufmann and Mohr, 1958), rubber (Boldingh, 1948), or silicones (Inouye *et al.*, 1955; Kritchevsky and Tiselius, 1951; Schlenk *et al.*, 1957), allows the fractionation of fatty acids according to the chain length. Mixtures of epoxy, hydroxy, and keto acids have been resolved (Kaufmann and Su Ko, 1962; Schafer *et al.*, 1960). Quantitation can be achieved by densitometry (Swartout *et al.*, 1960; Swartout and Herndon, 1965) or spectrometry of the eluted spots (Mangold *et al.*, 1955).

Partition, adsorption, or reversed-phase column chromatography have been successfully employed in fatty acid analysis; partition and reversed-phase chromatography give fractionations according to the molecular weight (Badami, 1964; Benton *et al.*, 1959; Blankenhorn *et al.*, 1961; Clasper and Haslam, 1957; Lis *et al.*, 1961; Kibrich and Skupp, 1959; O'Neal and Carlton, 1958; von Frankze, 1959), but critical pairs are eluted together (Frankel *et al.*, 1962; Savary and Desnuelle, 1953).

Silver nitrate-silicic acid columns, taking advantage of the formation of double-bond complexes, are useful in the fractionation of unsaturated mixtures (Anderson and Hollenbach, 1965; Barrett *et al.*, 1962; Bhatti and Craig, 1964; De Vries, 1963), or of geometrical isomers; ion-exchange argentated resins are effective in similar types of fractionation (Emken *et al.*, 1964; Sargent and Rieman, 1958; Sherma and Rieman, 1959; Scholfield and Emken, 1966; Wurster *et al.*, 1963). Quantitation in column chromatography has been attained by titrating the effluent, if free acids are used, by differential refractometry (Hirsch, 1963), or by more advanced procedures employing ionization flame detectors (Bombaugh and Little, 1964; Cotgreave, 1966; Hahti and Nikkari, 1963; James *et al.*, 1964).

TLC can be used to effect any desired separation of fatty acids and for preparation of small quantities of pure compounds. At the beginning, TLC was used as an absorption technique, and a variety of adsorbent materials (silica gel, alumina, kieselguhr) have been experimented with to fractionate compounds with different polarities (Mangold and Kammereck, 1962; Payne, 1964); the use of impregnated layers has enhanced its resolving power; all these techniques have been reviewed previously (Bobbitt, 1963; Mangold, 1965; Randerath, 1963; Stahl, 1964). In vegetable oil-fatty acid analysis, silica gel has been used to fractionate configurational isomers of hydroxy acids, epoxy acids (Morris, 1963; Morris and Hall, 1967), keto acids,

bromo derivatives of unsaturated fatty acids (Morris and Wharry, 1965; Roomi *et al.*, 1966; Sgoutas and Kummerow, 1963, 1964; Subbarao, 1962; Subbarao and Achaya, 1964), ozonides, and mercury acetate adducts (Jantzen *et al.*, 1961; Kuelliel, 1962; Mangold and Kammereck, 1961; Radin, 1965; Winterstein *et al.*, 1960); this last procedure is very effective in the fractionation of vinylogous groups (Kuelliel, 1962). Silica gel layers impregnated with boric acid, sodium borate, or sodium arsenite have a high resolving power for hydroxy and polyhydroxy acids and their configurational isomer mixtures (Applewhite, 1965; Lugay and Juliano, 1964; Morris, 1962). Unsaturated methyl esters are easily resolvable according to their unsaturation degree on silica gel or alumina impregnated with silver nitrate (Blank and Privett, 1963; De Vries, 1962; De Vries and Jurriens, 1963; Malins and Mangold, 1960; Matarese, 1964; Morris, 1962; Pallotta and Matarese, 1963; Paulose, 1966; Privett and Blank, 1963; Privett and Blank, 1964; Wood and Snyder, 1966; Privett *et al.*, 1963a).

Partition methods have been employed to resolve homologous series; reversed-phase systems, such as siliconized or hydrocarbon-impregnated plates have found wider applications for the same purpose (Carreau and Raulin, 1964; Hammond and Shone, 1964; Lugay and Julian, 1964; Kaufmann and Makus, 1960; Kaufmann *et al.*, 1961; Kaufmann and Khoe, 1962; Malins and Mangold, 1960; Ord and Bamford, 1966); critical pairs have been resolved by the use of two-dimensional systems, their polarity being changed by chemical methods after the first elution (Blank *et al.*, 1964; Privett and Blank, 1962; Purdy and Truter, 1962a). Quantitative analysis can be performed by measurement of the area (Purdy and Truter, 1962a,b) or optical density of spots, usually after charring with sulfuric-chromic acid mixtures (Blank *et al.*, 1964; Vioque and Vioque, 1964). Alternatively, elution from scraped portions of the plate permits the utilization of classical methods, such as gravimetry (Dunn and Robson, 1965), spectrometry (Vioque and Holman, 1962; Walsh *et al.*, 1965), GLC (Bowyer *et al.*, 1963a,b; A. N. Howard and Gresham, 1963; Privett and Blank, 1963); or radiometry (Collins and Somerville, 1964; Gunstone *et al.*, 1964; Snyder and Stephens, 1962; Yamada and Stumpe, 1964).

Vegetable oil fatty-acid analysis has been greatly advanced by the application of spectroscopy and spectrometry. Ultraviolet (UV) absorption spectroscopy can be applied to quantitation or structural analysis; this second application is limited to the study of conjugation that gives rise to characteristic absorption maxima in the UV re-

gion: dienes absorb at about 232 nm, trienes at 268 nm, tetraenes at 315 m μ . Double bonds conjugated with the carboxyl group also give rise to UV absorption (H. K. Black and Weedon, 1953; Herb, 1955; Holman and Hanks, 1955; O'Connor, 1955; Pitt and Morton, 1957); the position of the maximum is specific for any given compound. The intensity at the absorption maximum is used for quantitative analysis of fatty acid mixtures, provided that conjugated unsaturations are present. Alkaline isomerization of polyenic nonconjugated fatty acids, followed by UV spectrometry, as a means of measuring their concentration (American Oil Chemist's Society, 1946b), must not be overestimated (Vandenheuvel and Richardson, 1953).

Fatty acids and their esters have been studied extensively by means of infrared spectroscopy (IR) (Chapman, 1957, 1965; R. N. Jones *et al.*, 1952; R. N. Jones, 1962; O'Connor, 1956, 1960, 1961; Wheeler, 1954); the study of the unsaturation through IR, particularly of isolated or conjugated trans double bonds, has received considerable attention both for structure determination (Chapman, 1965; Heether *et al.*, 1951; Hopkins and Chisholm, 1962c; Jackson *et al.*, 1952) and quantitation (American Oil Chemist's Society, 1946c; Hopkins and Chisholm, 1962; Swern *et al.*, 1950). Polymorphism exhibited by long-chain acids gives rise to structurally spectral differences (Chapman, 1962). Data can be drawn from the $-\text{CH}_2-$ rocking modes (J. E. Jackson *et al.*, 1952); other functional groups in vegetable oil fatty acids, such as epoxy (Heether *et al.*, 1951) and cyclopropane (Numm, 1952), can be easily detected.

Nuclear magnetic resonance spectroscopy (NMR) gives signals for $-\text{CH}_2-$ and the $-\text{CH}_3$ groups of normal straight-chain acids (Hopkins and Bernstein, 1959; Hopkins, 1961, 1966); unsaturation and other functional groups are readily detected (Hopkins, 1961, 1966; Johnston *et al.*, 1964); the proton hydroxy groups in hydroxy acids give a sharp line whose position depends on the compound under examination (Hopkins, 1961). Cyclic groups such as cyclopropane in sterelic acid or cyclopentene in chaulmoogric acid have been studied by NMR (Hopkins and Bernstein, 1959). In a very few cases, NMR has been utilized in quantitative analysis (Johnston *et al.*, 1964; Storey, 1960; Wolff and Miwa, 1964).

The recent application of mass spectrometry has given a new approach to lipid chemistry; several reviews, to which the reader is referred, have appeared (Budzikiewicz *et al.*, 1964; Dutton, 1961; Hopkins, 1965; O'Connor, 1964; Refsgaard and Stenhagen, 1960, 1963). Location of double bonds in unknown molecules has been obtained

in particular cases by utilizing special techniques (Dinh-Nguyen *et al.*, 1959, 1961; Kenner and Stenhagen, 1964; Scholfield *et al.*, 1961c; Selke *et al.*, 1961). Mass spectra have proved useful in quantitative analysis of unsaturated and saturated acids (Hallgren *et al.*, 1957).

The combination of GLC and mass spectrometry has opened new perspectives because of the possibility of analyzing fatty acid mixtures or their derivatives, for structure assignment, prior to any fractionation (Leemans and McClosky, 1967).

Physical methods are indicative in structural analysis of fatty acids, but are seldom conclusive regarding a particular feature, such as the position of double bonds; chemical methods combined with TLC or GLC have been extensively used for that purpose, based on the oxidation and recognition of the fragments (Bergelson *et al.*, 1964; Edwards, 1966; Fedeli *et al.*, 1963; Gunstone and Sykes, 1962; Nickell and Privett, 1966; Privett *et al.*, 1963a; Stein, 1961).

2. Vegetable-Oil Fatty Acids

The fatty acid composition of seed oils is characteristic of the plant source; nevertheless, variations, due to climatic or seasonal conditions, are often observed.

Saturated and unsaturated acids, such as palmitic, stearic, palmitoleic, oleic, linoleic, and linolenic, are the major components of common seed oils; other saturated and unsaturated acids are often present as major or minor components; sometimes in specific seeds, unusual fatty acids predominate; the component acids of seed fats could themselves be a basis for a classification of plants (Hilditch and Williams, 1964). Table I shows the composition of some of the most common seed oils.

a. Saturated Fatty Acids. Among the saturated acids, lauric, palmitic, and stearic are the most common, palmitic acid being predominant over stearic acid in almost all the seeds examined (Ahrens *et al.*, 1959; Archer-Daniels-Midland, 1961; Earle *et al.*, 1961; Fisher and Broughton, 1960; Lea, 1929; Murti 1948). Short-chain fatty acids are very rare, but capric (*n*-decanoic) is, for instance, the major acid in *Cuphea* (Miller *et al.*, 1964b), in Ulmaceae and Lythrarieae seeds (Earle and Wolff, 1960; Hopkins and Chisholm, 1959; Miller *et al.*, 1964b; Sorensen and Soltof, 1958; T. L. Wilson *et al.*, 1960). Lauric acid is one of the major components of Lauraceae and Myristicaceae, together with myristic acid (Earle and Wolff, 1960; Miller *et al.*, 1964b); myristic acid predominates in Palmae (Collin and Hilditch, 1928; Longenecker, 1939; Mackie and Mieras, 1961). Arachidic

Table I
COMPOSITION OF SOME COMMON SEED OILS

Acids	Corn	Safflower	Rape	Linseed	Soybean	Cotton	Peanut	Olive	Palm	Sunflower	Sesame
14:0	-	Tr ^a	-	-	Tr	1	Tr	Tr	1	-	-
16:0	13	8	4	6	11	29	6	14	48	11	10
18:0	4	3	2	4	4	4	5	2	4	6	5
20:0	Tr	Tr	-	Tr	Tr	Tr	2	Tr	-	-	-
22:0	Tr	-	-	-	Tr	-	3	-	-	-	-
24:0	-	-	-	-	-	-	1	-	-	-	-
14:1	-	-	-	-	-	Tr	-	-	-	-	-
16:1	-	-	-	-	-	2	Tr	2	-	-	-
18:1	29	13	19	22	25	24	61	64	38	29	40
20:1	-	-	13	-	-	-	-	-	-	-	-
22:1	-	-	40	-	-	-	-	-	-	-	-
18:2	54	75	14	16	51	40	22	16	9	52	45
18:3	-	1	8	52	9	-	-	-	-	-	-

^aTr = trace.

Table II
UNUSUAL FATTY ACIDS OF SEED OILS^a

Scientific name	Formula	Trivial name	References
<i>Monounsaturated</i>			
<i>cis</i> -Doc-4-enoic	C ₁₀ H ₁₈ O ₂	—	1
<i>cis</i> -Dodoc-4-enoic	C ₁₂ H ₂₂ O ₂	—	1
<i>cis</i> -Tetradec-4-enoic	C ₁₄ H ₂₆ O ₂	—	2
<i>cis</i> -Hexadec-11-enoic	C ₁₆ H ₃₀ O ₂	—	3
<i>trans</i> -Hexadec-3-enoic	C ₁₆ H ₃₀ O ₂	—	4
<i>cis</i> -Octadec-6-enoic	C ₁₈ H ₃₄ O ₂	Petroselinic	5
<i>trans</i> -Octadec-3-enoic	C ₁₈ H ₃₄ O ₂	—	4
<i>cis</i> -Eicos-11-enoic	C ₂₀ H ₃₈ O ₂	—	6-8
<i>cis</i> -Eicos-5-enoic	C ₂₀ H ₃₈ O ₂	—	9
<i>cis</i> -Eicos-9-enoic	C ₂₀ H ₃₈ O ₂	Gadoleic	10, 11
<i>Cyclopropenyl</i>			
8-(2- <i>n</i> -Octylcycloprop-1-enyl)octanoic	C ₁₉ H ₃₄ O ₂	Sterculic	12, 13
7-(2- <i>n</i> -Octylcycloprop-1-enyl)octanoic	C ₁₉ H ₃₄ O ₂	Malvalic	12, 13
<i>Hydroxy</i>			
9-Hydroxy- <i>cis</i> -octadec-12-enoic	C ₁₈ H ₃₄ O ₃	—	14
9-Hydroxyoctadeca- <i>trans</i> -10- <i>cis</i> -12-dienoic	C ₁₈ H ₃₂ O ₃	—	15
9-Hydroxyoctadeca- <i>trans</i> -10- <i>trans</i> -12-dienoic	C ₁₈ H ₃₂ O ₃	Dimorphecolic	16
18-Hydroxyoctadeca-9-11,13-trienoic	C ₁₈ H ₃₀ O ₃	Kaulolenic	—
9,10-Dihydroxyoctadecanoic	C ₁₈ H ₃₄ O ₄	—	17
12,13-Dihydroxyoctadeca- <i>cis</i> -9-enoic	C ₁₈ H ₃₄ O ₄	—	18
9,14-Dihydroxyoctadeca-10,12-dienoic	C ₁₈ H ₃₂ O ₄	—	19, 20
14-Hydroxyeicos- <i>cis</i> -11-enoic	C ₂₀ H ₃₈ O ₃	Lesquerolic	21
11,12-Dihydroxyeicosanoic	C ₂₀ H ₄₀ O ₄	—	17
13,14-Dihydroxydocosanoic	C ₂₂ H ₄₄ O ₄	—	17
15,16-dihydroxytetra- <i>cis</i> -cosanoic	C ₂₄ H ₄₈ O ₄	—	17
<i>Epoxy</i>			
12,13-Epoxyoctadeca- <i>cis</i> -9-enoic	C ₁₈ H ₃₂ O ₃	Vernolic	22-25
9,12-Epoxyoctadecanoic	C ₁₈ H ₃₄ O ₃	—	5
9,10-Epoxyoctadeca- <i>cis</i> -12-enoic	C ₁₈ H ₃₂ O ₃	Coronanic	5

(Continued)

Table II (Continued)

Scientific name	Formula	Trivial name	Reference
15,16-epoxyoctadec- <i>cis</i> -9- <i>cis</i> -12-enoic	$C_{18}H_{30}O_3$	—	5
<i>Keto</i>			
15-Oxotetracos- <i>cis</i> -18- enoic	$C_{24}H_{44}O_3$	—	26
17-Oxohexacos- <i>cis</i> -20- enoic	$C_{26}H_{46}O_3$	—	26
9-Oxoctacos- <i>cis</i> - 12-enoic	$C_{28}H_{52}O_3$	—	19
-Oxoctadeca-9,11,13- trienoic	$C_{18}H_{28}O_3$	Licanic	20
<i>Diunsaturated</i>			
Deca- <i>trans</i> -2- <i>cis</i> -4-di- enoic	$C_{10}H_{16}O_2$	—	27, 28
Dodeca-2,4-dienoic	$C_{12}H_{20}O_2$	—	29
Octadeca- <i>trans</i> - 10- <i>trans</i> -12-dienoic	$C_{18}H_{32}O_2$	—	30-32
Octadeca- <i>trans</i> -9- <i>trans</i> -12-dienoic	$C_{18}H_{32}O_2$	—	33, 34
Eicosa- <i>cis</i> -11- <i>cis</i> -14- dienoic	$C_{20}H_{36}O_2$	—	35
Docosa- <i>cis</i> -13- <i>cis</i> -16- dienoic	$C_{22}H_{40}O_2$	—	36, 37
<i>Triunsaturated</i>			
Octadeca- <i>cis</i> -6- <i>cis</i> -9- <i>cis</i> -12-trienoic	$C_{18}H_{30}O_2$	—	38-40
Octadeca- <i>cis</i> -9- <i>trans</i> -11- <i>trans</i> -13- trienoic	$C_{18}H_{30}O_2$	Diostearic	41, 42
Octadeca- <i>cis</i> -9- <i>trans</i> - 11- <i>cis</i> -13- trienoic	$C_{18}H_{30}O_2$	Punicic	43-45
Octadeca- <i>cis</i> -8- <i>trans</i> - 10- <i>cis</i> -12-trienoic	$C_{18}H_{30}O_2$	—	44
Octadeca- <i>trans</i> -9- <i>trans</i> -11- <i>cis</i> -13- trienoic	$C_{18}H_{30}O_2$	—	31, 32, 34, 38, 41-44, 46-48
Eicosa- <i>cis</i> -5- <i>cis</i> -11- <i>cis</i> - 14-trienoic	$C_{20}H_{34}O_2$	—	35
<i>Tetraunsaturated</i>			
Octadeca- <i>cis</i> -9- <i>trans</i> -11- <i>trans</i> -13- <i>cis</i> -15- tetraenoic	$C_{18}H_{28}O_2$	Parinaric	49, 50
11- <i>cis</i> -Octadeca-6,9,12, 15-tetraenoic	$C_{18}H_{28}O_2$	—	40, 51

(Continued)

Table II (Continued)

Scientific name	Formula	Trivial name	References
<i>Acetylenic</i>			
Octadeca-9-ynoic	—	Stearolic	52, 53
Octadeca-6-ynoic	—	Tannic	54-56
Octadeca-trans-11-en-9-ynoic	—	Ximenyic or santalbic	57
Octadeca-cis-9-en-12-ynoic	—	Crepennyic	58, 59
Octadeca-trans-13-en-9,11-diynoic	—	Ximenic	—
Octadeca-17-en-9,11-diynoic	—	Isanic	60, 61
Octadeca-cis-13-en-9,11-diynoic	—	Bolekic	54, 56
8-Hydroxyoctadec-17-en-9,11-diynoic	—	Isanolic	62, 63

1-Toyama (1937); 2-Craig and Murti (1959); 3-De Tomas *et al.* (1963); 4-Kleiman *et al.* (1966); 5-Gunstone and Morris (1959); 6-McKinney and Jamieson (1936); 7-Hopkins (1946); 8-Hopkins *et al.* (1949); 9-Miller *et al.* (1964); 10-Bertam (1936); 11-Hopkins and Chisholm (1953); 12-Cornelius and Shone (1963); 13-Smith *et al.* (1951); 14-Gunstone (1952); 15-Badami and Morris (1965); 16-Earle *et al.* (1964); 17-Mikolajczak *et al.* (1965); 18-Scott *et al.* (1962); 19-Calderwood and Gunstone (1953); 20-Ahlers and Gunstone (1954); 21-Smith *et al.* (1961); 22-Gunstone (1954); 23-Morris and Wharry (1966); 24-Miwa *et al.* (1963); 25-Krewson *et al.* (1962); 26-Smith (1966); 27-Devine (1950); 28-Hilditch (1949); 29-Holman and Hanks (1955); 30-Hopkins and Chisholm (1962); 31-Hopkins and Chisholm (1964); 32-Morris and Marshall (1966); 33-Chisholm and Hopkins (1965); 34-Chisholm and Hopkins (1963); 35-Takagi (1964); 36-Hilditch *et al.* (1947); 37-Baliga and Hilditch (1948); 38-Riley (1949); 39-Roberts and Stevens (1963); 40-Smith *et al.* (1964); 41-Ahlers *et al.* (1953); 42-Bickford *et al.* (1953); 43-Farmer and Vandenhoevel (1936); 44-Hopkins and Chisholm (1962); 45-Chisholm and Hopkins (1964); 46-Ahlers and McFaggart (1954); 47-Ahlers *et al.* (1954); 48-Ahlers and Dennison (1954); 49-Takagi (1966); 50-Bagby *et al.* (1966); 51-Craig and Bhatti (1964); 52-Morris and Marshall (1966); 53-Gunstone and Subbarao (1966); 54-Gunstone and Sealy (1963); 55-Morris (1963); 56-Gunstone and Badami (1963); 57-Mikolajczak *et al.* (1963); 58-Hopkins and Chisholm (1966); 59-Ligtheim *et al.* (1952); 60-Steger and VanLoon (1940); 61-Doucet and Fauve (1942); 62-Riley (1951); 63-Kaufmann *et al.* (1951).

in *Chrysanthemum coronarium*; other epoxy acids isolated from seed oils are shown in Table II.

e. Keto Fatty Acids.—Three keto acids characterized by the $\text{CH}_2(\text{CH}_2)_n\text{CH}=\text{CH}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{R}$ grouping have been found in *Cuspidaria pterocarpa* seed oil (Brown and Farmer, 1965) (see Table II); a polyunsaturated keto acid, licanic, is present in oiti-

cica oil (*Licania rigida*) (Brown and Farmer, 1965), probably in the *cis*-9-*trans*-11-*trans*-13 form.

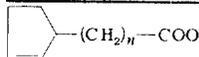
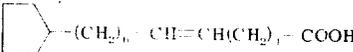
f. Cyclopentenyl Fatty Acids. In some genera of the Flacourtiaceae family, fatty acids characterized by the presence of a cyclopentenyl grouping have been found (Cole and Cardoso, 1938a,b, 1939a,b; Power and Gornall, 1904) which are active in the treatment of leprosy. The acids are shown in Table III. Chaulmoogric acid is the most important one, but hydnocarpic is also present in relatively large amounts; other acids are minor components of the *Hydnocarpus* seed fats.

g. Diunsaturated Fatty Acids. Among the diunsaturated acids, linoleic acid (octadeca-*cis*-9-*cis*-12-dienoic) deserves special mention because it constitutes the major fatty acid of many edible seed oils (Fisher and Broughton, 1960; Hilditch *et al.*, 1947) and is as ubiquitous as palmitic and oleic acids. Other diunsaturated acids are present in minor proportion in less common vegetable oils (see Table II).

h. Triunsaturated Fatty Acids. Linolenic acid (octadeca-*cis*-9-*cis*-12-*cis*-15-trienoic) is present as a major acid in a large number of vegetable oils (Cornelius and Shone, 1963), and sometimes it comprises, as in *Linum usitatissimum*, more than 50% of a seed oil. Other trienoic acids, some conjugated, have been found in vegetable oils (Takagi, 1964) (Table II).

i. Tetraunsaturated Fatty Acids. Tetraunsaturated fatty acids are rather rare in the vegetable kingdom. Parinaric acid is contained in the oil of *Parinarium laurinum*, *Impatiens*, and *Balsaminaceae* seeds. Another all-*cis*-octadecatetraenic acid (Table II) has been detected in *Nymphaea alba* seed.

Table III
CYCLOPENTENYL FATTY ACIDS IN FLACOURTIACEAE SEED OIL

Structure	Trivial name
	
$n = 0$	Aleprolic
$n = 4$	Aleprestic
$n = 6$	Aleprilic
$n = 8$	Alepric
$n = 10$	Hydnocarpic
$n = 12$	Chaulmoogric
	
	Gotlic

j. Acetylenic Fatty Acids. Acetylenic acids occur as glyceride components in two plant families, Santalaceae and Olacaceae; mono- and polyacetylenic as well as hydroxyacetylenic acids have been detected (Table II) in vegetable oils (Bu'Lock, 1964; Meade, 1957).

B. GLYCERIDES

Vegetable oils are mainly triglyceride mixtures resulting from the incorporation of component acids of seed fats, according to genetic rules, into the three positions of the glycerol molecule. The rules seem to apply over a wide range, giving rise to comparable glyceridic distributions.

1. Analytical Methods

Even if ester and iodine values are still important indices used for the characterization of oils, the problem of how triglycerides are composed and how fatty acids are distributed may be resolved only by other chemical and less traditional means. Different methods have been tried to reach this goal: Hilditch first proposed fractional crystallization and applied it to a large number of fats, collecting many interesting data (Hilditch and Williams, 1964). A somewhat different approach was used by the same author to determine the quantity of trisaturated glycerides by oxidizing the unsaturated ones to azelaoglycerides and then eliminating them by alkaline washing (Subbaram *et al.*, 1964).

All the older methods, very time-consuming and rather incomplete, have been replaced by more sophisticated approaches utilizing countercurrent distribution and chromatography.

Fractionation methods related to countercurrent distribution have been reviewed and automatic equipment and techniques described (Dutton and Scholfield, 1963; Dutton *et al.*, 1961; Dutton and Cannon, 1956; Scholfield *et al.*, 1961a,b); Scholfield and Dutton, 1958; Scholfield and Hicks, 1957; Therrault, 1953).

The composition of soybean, safflower, corn, and cocoa glycerides have been calculated (Butterfield, 1957; Scholfield and Dutton, 1959).

Chromatography on paper impregnated with silicic acid has been utilized to some extent to fractionate glyceride mixtures (Hamilton and Muldrey, 1961; Swartwout and Gross, 1957); fractionation according to the unsaturation degree has been achieved by elution with silver nitrate dissolved in methanol.

Silicic acid-silver nitrate columns (Barrett *et al.*, 1962; De Vries, 1962, 1963, 1964; Hirsch, 1961) have shown some advantage over the other techniques as a preparative tool of relatively high quantities of glycerides of similar unsaturation to be used for further resolution. Resolutions according to molecular weight occur on factice, a polymerized oil; partition columns have had very limited use (B. C. Black and Hammond, 1963; Trowbridge *et al.*, 1964).

More recently, fractionation according to unsaturation has been obtained by TLC on silver nitrate-impregnated plates (Barrett *et al.*, 1962, 1963; De Vries, 1962; De Vries and Jurriens, 1964; Jurriens *et al.*, 1964a,b), and quantitation has been achieved by densitometry (Purdy and Truter, 1962a,b) or by glycerol determination (Jurriens *et al.*, 1964a) colorimetry (Hirsch, 1961) or GLC of the methyl esters in presence of a suitable standard (Blank *et al.*, 1965).

Reversed-phase TLC fractionates triglycerides according to their molecular weight and has been used alone, or with silver nitrate complex formation (Ord and Bamford, 1967), to effect separation of triglycerides. GLC on SE 30 or JXR (Huebner, 1961; Kuksis and McCarthy, 1962; Kuksis, 1963, 1965; Litchfield *et al.*, 1965; Kuksis and Ludwig, 1966) also permits fractionations according to the molecular weights of the triglycerides and has been used, together with TLC on silver nitrate-impregnated layers (Fedeli *et al.*, 1965). Under optimal operating conditions, triglycerides with a molecular weight as high as that of trierucin can be quantitatively analyzed (Jurriens and Schouten, 1965); this offers a valuable means for determination of the structure of glycerides in relation to the study of distribution rules (Harlow *et al.*, 1965). None of the outlined techniques alone can give a complete knowledge of the distribution of fatty acids in triglycerides; nevertheless, their combination has found a broad application.

At present, the most complete analysis that can be done on vegetable oil triglycerides involves the acquisition of the following data (Coleman, 1961, 1965b; Jurriens and Kroesen, 1965; Gunstone *et al.*, 1964; Litchfield *et al.*, 1964; Litchfield and Reiser, 1965; Perkins and Hanson, 1965; Vander Wal, 1960): (a) overall fatty acid composition by GLC; (b) fractionation of glycerides according to their unsaturation, coupled with subsequent fractionation, according to their molecular weight; (c) distribution of the fatty acids in the 1,3-positions, and in the 2-position of the glycerides: this point can be studied by selective hydrolysis with pancreatic lipase (Mattson and Volpenheim, 1961; Savary *et al.*, 1957). The fatty acid distribution at

the 1-position of glycerol, independently of that of 3-position, can be known by more sophisticated methods (Brockerhoff, 1965; Brockerhoff and Yurkowski, 1966; Yurkowski and Brockerhoff, 1966).

The structure of the triglycerides has been studied by means of X-ray diffraction (Chapman, 1957, 1964) and mass spectrometry (Dutton, 1961; Ryhage and Stenhagen, 1960, 1963).

The method outlined here has been widely used to analyze the triglyceride of many vegetable oils; palm (Jurriens *et al.*, 1964a,b), cocoa (Jurriens and Kroeser, 1965; Mattson and Volpenhein, 1961), groundnut (Culp *et al.*, 1965), rapeseed (Grynberg *et al.*, 1966; Jurriens and Schouten, 1965), corn and sunflower (Gunstone and Qureshi, 1965), linseed (Vereshchagin and Novitskaja, 1965a,b), and olive (Vioque *et al.*, 1964).

Less common vegetable oils have been studied in an attempt to find the distribution rules which govern the biosynthesis of triglycerides (Blank *et al.*, 1965; Gunstone *et al.*, 1965b; Gunstone and Padley, 1965; Jurriens and Kroesen, 1965; Litchfield *et al.*, 1964; Kaufmann and Das, 1962; Kaufmann and Wessels, 1964; Ord and Bamford, 1967).

2. Distribution Theories

Several reviews have already appeared on this subject since Hilditch's monumental work (Hilditch and Williams, 1964). We wish only to point out how the new research methods developed in the last ten years have permitted a much more detailed knowledge of the distribution of the fatty acids in vegetable oil triglycerides.

Pancreatic lipase hydrolysis has proved (Coleman, 1963a; Fedeli *et al.*, 1965; Gunstone and Padley, 1965; Mattson and Volpenhein, 1961; Vander Wal, 1960) that acids are not randomly distributed on the three positions of glycerol; with very few exceptions, the 2-position is predominantly occupied by unsaturated acids, whereas the saturated and C_{20-22} monounsaturated acids are predominantly distributed in the 1,3-positions of glycerol.

The so-called "random distribution hypothesis" originally proposed by Hilditch's co-workers has, therefore, been abandoned in favor of several "restricted random" theories (Gunstone, 1962; Kartha, 1953; Lands *et al.*, 1966; Subbaram and Youngs, 1964; Youngs, 1961; Youngs and Subbaram, 1964; Vander Wal, 1960); the 1,3-random-2-random hypothesis of Vanderwal considers that saturated acids are mainly random distributed on the 1- and 3-positions, whereas unsaturated ones are random distributed on the 2-position. Evidence for the validity of this hypothesis has been given by many

authors utilizing lipolysis data which agree in the majority with the data obtained by other methods (Coleman, 1965a; Gunstone and Subbarao, 1966; Vander Wal, 1965). Small departures have been noted for palm oil and some unusual fats that do not conform to the theory. Gunstone's theory also shows a good agreement with the experimental data.

Brockerhoff (1965), Lands and co-workers (1966), and Yurkowski and Brockerhoff (1966) have proved that 1- and 3-positions are not completely symmetrical.

Assuming that the 1,3-random-2-random theory is valid, it would be possible to calculate the types and amount of each triglyceride present in the oil and the fatty acid distribution.

As an example of the conclusions that can be drawn from the combined application of chromatographic and enzymatic techniques, see Table IV, where data are reported on the fatty acid distribution in olive and tea-seed oils (Fedeli and Jacini, 1967).

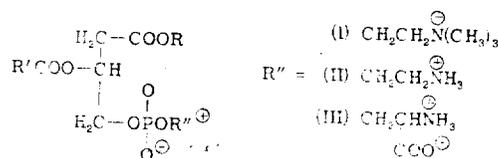
III. Other Lipids

A. PHOSPHOGLYCERIDES

Phosphoglycerides are widespread in vegetable oils. Wittcoff's and Dittmer's monographs (Dittmer, 1960; Wittcoff, 1951) cover the subject up to 1960; after that date, a marked advance was made possible by the introduction of new chromatographic methods, which in this case also permit structural analysis.

1. Structure of Phosphoglycerides

Seeds with high oil content usually show the presence of appreciable quantities of these compounds as complex mixtures of polar materials; phosphatidylcholine (I) (or lecithin) and phosphatidylethanolamine (II) (or cephalin) are widely distributed; both the compounds have the 1,3-glycerophosphate structure, like other phospholipids thus far studied.



Phosphatidylserine (III) is usually present in seeds in a lower concentration than the other two; the serine moiety shown (III) has the L-structure.

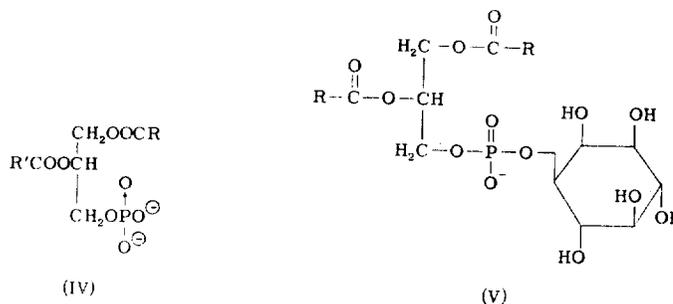
Table IV
FATTY ACIDS DISTRIBUTION IN OLIVE AND TEA-SEED OILS GLYCERIDES^{a,b}

Number of double bonds per triglyceride molecule	Olive oil		Tea-seed oil		
	Fatty acids distribution	% amount on the oil	Fatty acids distribution	% amount on the oil	
0	—	—	—	—	
1	POP	2.9	POP	2.08	
	PPO	0.63	PPO	0.34	
	POS	0.47	POA	0.98	
	SPO	0.14	SPO	0.01	
	PSO	0.25	PSO	0.02	
	SOS	0.18	SOS	0.02	
	SSO	0.01	SSO	Trace	
	N.D.	0.23	N.D.	Trace	
	2	PLP	0.08	PLP	0.01
		PPL	1.18	PPL	Trace
POO		18.40	POO	2.58	
POO		2.27	OPO	Trace	
PLS		0.12	PLS	0.2	
LPS		0.70	LPS	0.04	
LSP		0.06	LSP	0.04	
SOO		5.10	SOO	0.69	
OSO		0.06	OSO	—	
SLS		0.03	SLS	Trace	
SSL		—	SSL	—	
N.D.		2.16	N.D.	Trace	
3		PLO	0.17	PLO	0.15
	POL	5.90	POL	2.75	
	OPL	0.94	OPL	1.45	
	SLO	2.40	SLO	0.00	
	LSO	0.72	LSO	0.15	
	SOL	1.28	SOL	1.07	
	OOO	43.50	OOO	63.6	
	N.D.	0.61	N.D.	8.0	
	4	PLL	0.38	PLL	0.00
		LPL	0.17	LPP	0.25
OOL		6.76	OOL	9.95	
OLO		3.50	OLO	5.15	
SLL		0.20	SLL	Trace	
ASL		0.27	LSL	Trace	
N.D.		0.1	N.D.	0.33	

^aAccording to Fedeli and Jacini, 1967.

^bP = Palmitic acid; S = stearic acid; O = oleic acid; L = linoleic acid; N.D. = other acids.

Phosphatidic acids (IV) are probably present among the phospholipid pool of seed oils in trace amounts even if it may constitute an artifact from the phospholipase D action on the other phospholipids (Kates, 1956, 1957; Wheelidon, 1960).



Cardiolipin (1,3-diphosphatidylglycerol) has been found in soybean lipids (Benson and Strickland, 1960); it is probably the precursor of the 1,3-diglycerophosphoryl glycerol found in corn and other vegetable oils (Okubara and Nakajama, 1955); phosphatidylinositol (V) has been isolated from soybean and peanut phosphatides (Hawthorne *et al.*, 1960; Lepage *et al.*, 1960; Okubara and Nakajama, 1955).

2. Analytical Methods

The chemical analysis of phospholipids has greatly improved since the introduction of chromatographic methods; excellent reviews have appeared (Hanahan, 1960; Marinetti, 1962; Pries *et al.*, 1966; Rouser *et al.*, 1965a; Wittcoff, 1951) describing methods and techniques.

Extraction (Entenman, 1957; Letters, 1966; Sperry, 1955), removal of nonlipid contaminants (Letters, 1966; Rouser *et al.*, 1965a; Siakotos and Rouser, 1965), and separation from other lipids are the initially important steps in the analysis, followed by several possible procedures, mostly based on chromatography.

Column chromatography has been extensively used: alumina has found limited utilization because of the formation of artifacts, but silicic acid, cellulose, and diethylaminoethyl cellulose (DEAE) have met general requirements (Camejo, 1965; Horiguchi and Kaudatsu, 1960; Letters, 1966; Rouser *et al.*, 1961, 1965a,b). Rouser introduced silicic acid columns containing silicate and water to effect special separations, for instance, of phosphatidylethanolamine from phosphatidylserine (Camejo, 1965; Rouser *et al.*, 1961b). Magnesium sili-

cate has been used to some extent to free polar from contaminant neutral lipids (Radin *et al.*, 1955; Rouser *et al.*, 1961a, 1963).

TLC, since its introduction in phospholipid analysis, has become popular because of many advantages, such as improved resolution and availability of color reactions (Kaufmann *et al.*, 1963; Rouser *et al.*, 1961a), or charring methods for the quantitative determination by densitometry (Blank *et al.*, 1964; Privett and Blank, 1963; Rouser *et al.*, 1964); complex mixtures of polar eluents are required for good separations (H. Wagner, 1960; Weicher *et al.*, 1960). Impregnation of plates with sodium borate, carbonate, or acetate can furnish some improvement (Skipski *et al.*, 1962, 1963). Two-dimensional TLC (Lepage, 1964; Letters and Brown, 1944; McKillican, 1963; Skidmore and Entenman, 1962), better than monodimensional, can give a complete resolution of phospholipids, suitable for characterization and quantitative analysis (Malins, 1966; Rouser *et al.*, 1965a). Complex mixtures are resolvable by combining column and TL chromatography; Rouser *et al.* (1965a,b) recommended DEAE, magnesium silicate, or silicic acid columns, in combination with TLC, as being particularly useful because of their precision and speed. TLC has been used to fractionate phospholipids, according to the unsaturation degree, on argentated plates (Addison, 1965; Kaufmann *et al.*, 1963). Paper chromatography at present has a very limited application, but has been extensively employed in the past. Structural analysis of pure phospholipid fractions involves the combination of several analytical procedures based on stepwise specific hydrolysis to show the nature and position of the acyl groups and their distribution on the phosphoglyceride molecule (Dawson, 1954, 1960; De Haas *et al.*, 1965; De Koning and McMullan, 1965; Renkonen, 1965, 1966; Nutter and Privett, 1966a,b); the other constituents are analyzed after isolation by conventional means (Dittmer, 1960; Wittcoff, 1951). The total phosphoglyceride content of vegetable oils is usually estimated by phosphorus determination and then multiplying by an appropriate conversion factor (25 for most of the phosphoglycerides; even small quantities of phosphorus have been estimated by spectrophotometric methods (Long, 1943; Long and Staples, 1961).

B. NONGLYCERIDIC COMPONENTS

1. Introduction

All lipids other than glycerides, partial glycerides and phospholipids, are included in this section. The nonglyceridic components

(NGC) constitute a small fraction of vegetable oils, seldom over 5%, and generally less than 2%. The information we actually have about them is still very incomplete. In spite of the limited amounts present, the importance of the NGC should not be overlooked; many of them form a characteristic and quite distinct fraction (e.g., terpene alcohols); others are known to possess antioxidant, flavoring, or pigmentary functions. Toxic NGC are present in many vegetable oils, and they should be removed if the oil is destined for food purposes; for example, the gossypol group in cotton oil (gossypol, diaminogossypol, gossypurpurin) (El-Nockrashy *et al.*, 1963) and the sulfurated glucosides present in the seeds of Cruciferae, Resedaceae, Copparidaceae families, which through enzymatic hydrolysis lead to the goitrogenic action of isothiocyanates and oxazolidinethiones (Clements and Wishart, 1956; Hecker *et al.*, 1965; Kjaer *et al.*, 1959; Kjaer, 1962).

It is possible that toxic substances, such as saponins, are also present in soybeans and peanut seeds (Dieckert *et al.*, 1959; Gestetner, 1964). Various toxic substances have been detected in little-known oils, but the study of them has not gone beyond the detection of their toxicity (Holmes and O'Connor, 1961; Kyrdzheiva, 1956). Various authors believe they have identified among the NGC some hypocholesteremic factors (Nath *et al.*, 1959; Wilkens and De Wit, 1962), but on the whole this still requires confirmation. A large part of the information provided in the literature, prior to the extensive use of TLC and GLC, has exclusively a qualitative and informative significance; in fact, the major progress in NGC knowledge dates from the intensive use of chromatographic methods. It is impossible to list all the substances which have been identified (and sometimes not surely identified) in vegetable oils. In many cases it is not absolutely certain that they are natural substances rather than denaturation products thereof. Such is the case, for example, of aldehydes, which have been repeatedly identified in oils, but still not definitely demonstrated as being primary substances and not due to enzymatic or autoxidation degradations (Hoffmann and Keppler, 1960; Keppler and Beerthuis, 1962).

We are purposely excluding in the following discussion those substances which are surely artifacts, due to enzyme actions, such as substituted furans (Chang *et al.*, 1967) and the substances deriving from alterations undergone by the NGC owing to some industrial refining treatment (Rousser, 1966). It rarely occurs that a NGC is directly isolated from a vegetable oil; this may happen only for components present in rather abundant amounts, or possessing peculiar

chemical features, such as palm oil carotenes (Arpino *et al.*, 1960), sesame oil sesamolins (Budowski, 1964), colza oil alkyl thiocyanates (Kjaer *et al.*, 1959; Kjaer, 1962), but they are exceptions; as a rule their study requires a preliminary concentration. Different enrichment methods have been used: molecular distillation, extraction with more or less selective solvents, the various chromatographic techniques. None of these methods, however, has proved as practical as the preparation of the unsaponifiable; this is a means which permits the easy collection of comparatively large quantities of nonglyceridic lipids. Obviously not all the NGC present in a unsaponifiable are natural substances (i.e., primary); the unsaponifiable fraction consists of what remains of the NGC after a nonpreservative chemical treatment. Here it must be noted that to obtain the NGC from a vegetable oil it is often impossible to apply entirely the methods used to study the lipids of organs or tissues (vegetables or animal) (Fillerup and Mead, 1953; Malins and Mangold, 1960; Mangold, 1964; Mangold and Malins, 1960); here, in fact, the terpenoids are present in amounts comparable to those of other oils, whereas a vegetable oil is composed of over 95% triglycerides and free fatty acids (FFA), with modest amounts of NGC. It is for this reason that the knowledge we possess of vegetable oil NGC is seldom based on quantitative evaluations.

We will attempt to list the NGC classes definitely identified in vegetable oils, in order of increasing polarity: hydrocarbons (normal and iso- and ante-isoparaffins, polycyclic aromatics), squalene, carotenoids, aliphatic alcohols and waxes, chlorophylls, acyclic and cyclic terpene alcohols (as such, or as esters of fatty or aromatic acids), xanthophylls, tocopherols (as such, or as esters), flavors (Bergelson *et al.*, 1966; Mitsuda *et al.*, 1958), coumarins (Benson *et al.*, 1962), phenolic compounds (Privett *et al.*, 1963b; Vazquez Romero *et al.*, 1961), sterols (as such, or as esters or glycosides) (Lepage, 1964), aldehydes, aliphatic diol esters (Bergelson *et al.*, 1966), phenolic acids (esterifying terpene alcohols), and terpenic acids (Vazquez Romero, 1964).

These various components will be described in Sections 2 and 3, according to their occurrence.

We cannot exclude the fact that vegetable oils may also contain other lipid classes, such as those identified in photosynthetic leaves and tissues; the same techniques to be followed in order to obtain an oil from a seed or a fruit make perfectly permissible the hypothesis that the oil may act as solvent of substances present in other parts of the seed; thus it cannot be excluded that the following may be

present in a vegetable oil: phytosphingolipids (Carter *et al.*, 1961, 1963; Prostenik *et al.*, 1958; Van Handel, 1953), sulfolipids (Benson *et al.*, 1962; Benson, 1963; Collier *et al.*, 1963; Goldberg, 1961; Kates, 1960; Klopfenstein and Shigley, 1966), galactosyl lipids (O'Brien and Benson, 1964), cerebrosides (Carter *et al.*, 1961), lipoproteins (Kaufmann, 1957; Zill and Harmon, 1961), even if, so far, none of these compounds has been materially identified in a vegetable oil.

Some of the chemical types which are definitely present in oils are represented by various series of homologs, others by a unique individual one.

2. Components of the Unsaponifiable Fraction

The major part of the research on NGC has been conducted to date utilizing unsaponifiables as raw material; as we have already noted, these cannot contain the total NGC listed above; in unsaponifiables, components cannot be present which give water-soluble alkaline salts or which are altered during saponification.

A unsaponifiable can be directly obtained from the oil, or after being previously enriched by various means (molecular distillation, crystallization, countercurrent fractionation, methanolysis and methyl esters distillation); the unsaponifiable composition is somewhat different, quantitatively at least, according to the enrichment system used; many unsaponifiable constituents, in fact, are present in the oil in different forms which are affected in various ways by a particular method of enrichment.

A minor, but not negligible, influence on the amount and components of a unsaponifiable is also exerted by the manner in which the oil is obtained; if by pressure alone, it will always contain less unsaponifiable than the oils obtained by extraction with a solvent which also entrains or dissolves the lipids-proper of the vegetable (such as the waxes of hulls).

The quantity and composition of an unsaponifiable depends in the first place on the oil type, i.e., on the botanic species of the seed and fruit; coffee oil gives up to 6-9% unsaponifiable; avocado oil, 6-7%; and pressure-olive oil, up to 1.0%.

The unsaponifiable, moreover, is present in different qualities and quantities according to the seed portion from which the oil is derived; in the few cases studied it has been noted that the oil obtained from cotyledons is almost free of NGC, whereas the oil obtained from embryos is rich in NGC; our knowledge in this respect is practically confined to soybeans (Fedeli *et al.*, 1966b), peanuts (Fedeli *et*

al., 1968), and cocoa beans (Capella *et al.*, 1964a). We know little also about the modifications undergone by the unsaponifiable components during maturation of an oil-bearing seed or fruit and during its germination (Dubinskaja, 1959; Fedeli *et al.*, 1968; Vazquez Romero *et al.*, 1965).

The fractionation technique for unsaponifiable components, always based on chromatographic methods, has recently been greatly improved. Because of the complex composition of most unsaponifiables studied, several steps are required before compounds are sufficiently pure to enable their identification directly by traditional chemical means, or by spectrographic methods. The method giving the best results, especially preparative, consists of a series of adsorption chromatographies on silica or alumina columns or, less frequently, on Florisil or other inert adsorbents. The use of ionic adsorbents (DEAE and TEAE) or of exchange-resins offers no advantage.

A series of increasing polarity solvents or mixtures is used, thus eluting successively compound groups also of increasing polarity. The first separation permits the resolution of the nonsaponifiable into 4 or 5 fractions, more or less well defined (Capella *et al.*, 1960).

Neither countercurrent fractionation (Therrault, 1963), although frequently applied, nor partition chromatography, seems to be a method generally used. It is also possible, in lieu of column separation, to fractionate the unsaponifiable on silica gel plates (TLC) (Privett *et al.*, 1965; Rouser *et al.*, 1965a; Vazquez Romero *et al.*, 1961), usually monodimensional; here, however, the use of an eluent alone and the small unsaponifiable quantities fractionable on a plate make the task more difficult. With regard to unsaponifiables, the bidimensional TLC (Beiss, 1964) offers no substantial benefits. The efficiency and trend of column fractionation can be monitored by various methods, generally by TLC on a fraction at intervals.

Having obtained a series of groups of substances with sufficiently distanced polarity, it is necessary to have recourse to subsequent fractionation by TLC, employing solvents or solvent mixtures of extremely calibrated polarity (Malins and Mangold, 1960; Mangold and Malins, 1960); the single fractions are thus "cleaned" and resolved, in turn, into subgroups, or directly into substances having a high degree of purity.

It is often advisable, rather than operating directly on column-derived fractions, to reduce their polarity by making an appropriate derivative; for instance, acetylating (in pyridine with acetic anhydride) the entire fraction and then separating the obtained acetyl de-

rivatives by TLC. The spectrographic methods (UV, IR, NMR, MS) applied to the individual TLC-obtained fractions may now begin to furnish useful information: for example, GLC analysis on a proper polarity column, generally at programmed temperatures. TLC separation on silver-coated plates; at this stage of fractionation, is not usually applied (Morris, 1966).

There is no universal method for the study of NGC's (Mangold, 1964; Rouser *et al.*, 1965a); remarkable variations, in fact, are required, depending on the prevailing component type present, or on the purpose to be attained; very often TLC separations must have countercurrent separations, crystallizations, or fractional distillations, saponifications or hydrogenolyses coming between, in order to remove the protective groups introduced into the NGC (Privett *et al.*, 1965). Table V summarizes the unsaponifiable composition of some of the best-known vegetable oils.

We will now briefly describe the principal NGC categories as well as their constituents obtainable from unsaponifiables.

a. Hydrocarbons. The classes definitely represented in vegetable oils include paraffins (normal or branched), terpenoids (squalene and homologs), polycyclic aromatics; the presence of high-molecular weight and low-unsaturation-degree olefins, such as zamene (Tsujimoto, 1935) and gadusene (Nakamiya, 1935) and the presence in olive oil of C_{13} - C_{28} olefins, reported by Marcelet (1936), need to be confirmed.

All vegetable oils seem to contain paraffin hydrocarbons (Kaufmann, 1958) whose genesis is still obscure; paraffin $C_{29}H_{60}$ has been known since 1948; the paraffins contained in a vegetable oil are very numerous (Capella *et al.*, 1960, 1963a; Jacini, 1963) from 25 to 45 chemical individuals of increasing molecular weight from $C_{11}H_{24}$ to $C_{35}H_{72}$. Odd C-number terms seem to prevail over even

Table V
UNSAAPONIFIABLE COMPOSITION OF SOME WELL KNOWN VEGETABLE OILS

Oils	Hydrocarbons	Squalene	Aliphatic alcohols	Terpenic alcohols	Sterols
Olive	2.8-3.5	32-50	0.5	20-26	20-30
Linseed	3.7-14.0	1.0-3.9	2.5-5.9	29-30	34.5-52
Tea-seed	3.4	2.6	-	-	22.7
Soybean	3.8	2.5	4.9	23.2	58.4
Rapeseed	8.7	4.3	7.2	9.2	63.6
Corn	1.4	2.2	8.9	6.7	81.3

ones; more than two homologous series (Jarolinek *et al.*, 1964), normal and iso, are probably represented; the hydrocarbon $C_{29}H_{60}$ is one of the main components (Capella *et al.*, 1963a; Evans *et al.*, 1964).

The identification of the numerous paraffins detected can be made by comparing the ratios of retention times in isotherm GLC versus C atom number. With unknown hydrocarbons and synthetic homologous series having a similar structure, GLC allows no differentiation when the ratio is identical or extremely close for two series of different structures. For example, with branched paraffins, GLC cannot establish whether they belong to the iso series, $(CH_3)_2 CH-R$, or to the ante-iso $(C_2H_5CH_2) CH-R$. The presence is not excluded, however, of other paraffin series, possibly also branched.

More interesting for their genesis and as precursors of sterols are the terpene hydrocarbons, among which the squalene $C_{30}H_{50}$, the only one surely identified (Fitelson, 1946; Taulfel *et al.*, 1940; Thorjamarson and Drummond, 1935), is present in varying amounts in all the vegetable oils studied; olive oil appears to contain a major quantity (400-700 mg/liter) (see Table V).

The presence in oils of hydrocarbon homologs of squalene, although suggested, has not yet been confirmed (Gracian, 1961). Recently, the presence of polycyclic aromatic hydrocarbons (Jung and Morand, 1964; J. W. Howard *et al.*, 1965a) has been demonstrated in many vegetable oils. First revealed through their fluorescence spectra (Ciusa *et al.*, 1965) and afterward identified with greater precision (J. W. Howard *et al.*, 1965, 1966b), their quantity is in the order of a few units per billion (10^{-3} ppm).

b. Aliphatic Alcohols. It cannot be said whether these are present as such in the unsaponifiable or whether they derive from waxes; the latter hypothesis is most probable.

The unsaponifiable alcohols are the water-insoluble, higher ones; a series of at least 8 components has been identified, from C_{22} to C_{32} (Jacini, 1963; Jacini and Fedeli, 1967); no branched aliphatic alcohols have been detected so far, nor any unsaturated alcohols, although their presence is highly probable. Secondary normal alcohols have been isolated (Chibudi *et al.*, 1934).

The identification of alcohols by GLC is a rather simple operation.

Waxes, as esters, theoretically should not be present in unsaponifiables; to isolate them as such, other techniques are required (see Section III, B, 3).

c. Carotenes. These are difficult to find intact in a unsaponifiable fraction. Generally, the saponification treatment alters them, re-

dering them unrecognizable; only by operating on small quantities of oil in a nitrogen atmosphere is it possible to avoid, in part, their decomposition (Capella *et al.*, 1963b).

Consequently, it is advisable to attempt to separate carotenes directly from the oil, preferably by a particular enrichment procedure (Jacini and Fedeli, 1967). For certain industrial purposes, adsorption methods (Arpino *et al.*, 1960) may be used, but only when starting with oils very rich in carotenes, i.e., palm oil, and without attempting to recover them quantitatively. Vegetable oils contain β -carotene almost exclusively, which can be isolated together with xanthophylls (Capella *et al.*, 1963b; Pelt, 1961), and subsequently separated from them by TLC.

d. Terpene Alcohols. The study of terpene alcohols dates back to 1957, somewhat facilitated by the knowledge already acquired about analogous components of essential oils and natural resins. Terpene alcohols are present in vegetable oils partly in free form and partly as esters (Jacini and Fedeli, 1967); the acids which esterify them are still little known; so far, fatty acids have been identified which are similar to those of vegetable oils (Jacini and Fedeli, 1967) and ferulic acid (Kato *et al.*, 1961; Shimizu *et al.*, 1957); in unsaponifiables, obviously, only free terpene alcohols are present.

The components identified to date belong exclusively to three series; those of acyclic terpenic, and of cyclic di- and triterpenic. The study and resolution of terpene alcohols from an unsaponifiable is in general not simple; the compounds present may be numerous and always have very similar features; also it is often difficult to free them of higher aliphatic alcohol traces. The number of components present can be precisely known through GLC or TLC on silver-coated plates (Capella *et al.*, 1963c,d), and they can be identified by the traditional means of investigation used for natural substances. Two acyclic terpene alcohols definitely identified at present are phytol and geranylgeraniol. Phytol very probably is not present as such in oils, but derives from a chlorophyll; it should then be present in all the unsaponifiables. Geranylgeraniol was separated for the first time from linseed oil (Fedeli *et al.*, 1966a), in which it is probably present in free form. This terpene alcohol is interesting as a likely precursor of di- and tricyclic diterpenes and of some carotenes, including lycopene (Demole and Lederer, 1958; Popjak, 1965; Ruzicka, 1953; J. Wagner, 1932).

The group of cyclic terpene alcohols comprises a much greater number of members, according to the botanic species (see Table VI). So far, 13 have been found in one oil (rapeseed oil); coffee-bean oil

Table VI
NUMBER OF CYCLIC TERPENE ALCOHOLS IN VARIOUS VEGETABLE OILS

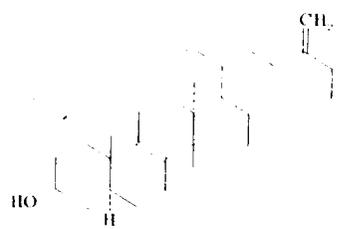
Alcohol	Almond	Apricot	Avocado	Banana											
Cycloartenol															
24-Methylcycloartanol															
24-Ethylcycloartanol															
24-Propylcycloartanol															
24-Butylcycloartanol															
24-Pentylcycloartanol															
24-Hexylcycloartanol															
24-Heptylcycloartanol															
24-Octylcycloartanol															
24-Nonylcycloartanol															
24-Decylcycloartanol															
24-Undecylcycloartanol															
24-Dodecylcycloartanol															
24-Tridecylcycloartanol															
24-Tetradecylcycloartanol															
24-Pentadecylcycloartanol															
24-Hexadecylcycloartanol															
24-Heptadecylcycloartanol															
24-Octadecylcycloartanol															
24-Novecylcycloartanol															
24-Triacontylcycloartanol															

contains three members only (Kaufmann *et al.*, 1964); other oils, never fewer than five (Fedeli *et al.*, 1966c). Table VII lists the structure of those definitely identified up to now.

The most widespread of these alcohols seems to be cycloartenol, first identified in a series of nonoleaginous botanic species (*Artocarpus integrifolia*) (Barton, 1952), *Strachnos nux vomica* (Bentley *et al.*, 1955), *Euphorbia handiensis* (Gonzales and Breton, 1953), *Euphorbia balsamifera* (Chapon and David, 1952), and finally in rice oil (Shimizu *et al.*, 1957) and in linseed oil (Capella, 1961). Cycloartenol has always been found in all the vegetable oils in which it has been sought (Jacini and Capella, 1961; Fedeli *et al.*, 1966c). The homolog 24-methylcycloartanol, first isolated from rice oil (Shimizu *et al.*, 1957), then from wheat germ and soybean oil (Tamura *et al.*, 1962b), and from linseed and olive oil (Picchi *et al.*, 1966), is also so diffuse that it suggests the same biological source as cycloartenol.

The group of cyclic triterpene alcohols of vegetable oils, such as may be obtained from a column or a plate, always presents a yellow-

Table VII



Euphorbol



Cyclotaudenol



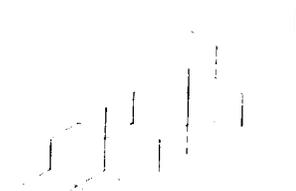
α -Myrrin



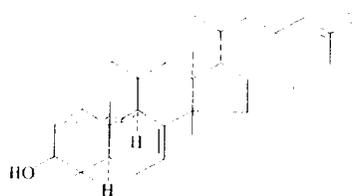
β -Myrrin



Cycloartenol



24-Methylcycloartenol



Butyrospermol

brownish Liebermann-Burchard reaction, remarkably different from that exhibited by sterols; terpene alcohols having a cyclopropane ring in 9, 19, such as cycloartenol, also give a yellow reaction with tetranitromethane. Some members, moreover, yield a particular reaction, the Fitelson reaction (Fitelson, 1945, 1946). Proposed in the past as specific of tea-seed oil (American Oil Chemist's Society, 1946a), it has been recently recognized (Fedeli *et al.*, 1966b; Jacini, 1963; Capella *et al.*, 1963e), however, that many terpene alcohol groups derived from vegetable oils contain a Fitelson-positive component: it can be inferred, then, that the reaction "specificity" (Condorelli and Gurrieri, 1960), is exclusively due to the particularly abundant amount in which a Fitelson-positive member is present; in the case of tea-seed oil, this member is butyrospermol (Capella *et al.*, 1963e), present also in very small quantities in olive and oiticica oils. Linseed oil also contains a Fitelson-positive terpene alcohol whose structure is still unknown (Jacini *et al.*, 1971). The Fitelson reaction is probably attributable to the side-chain orientation at 17, having an α -configuration. All Fitelson-positive compounds (euphol, butyrospermol, tyrucalol, euphorbol), belong to the euphan series, whereas terpene alcohols having the β -side chain (such as cycloartenol) are Fitelson-negative.

No reliable data exist confirming whether or not there are combination products formed between cyclic triterpene alcohols and digitonin; if they form, as seems possible, they would be more easily hydrolyzable than sterol digitonides (Jacini and Capella, 1961).

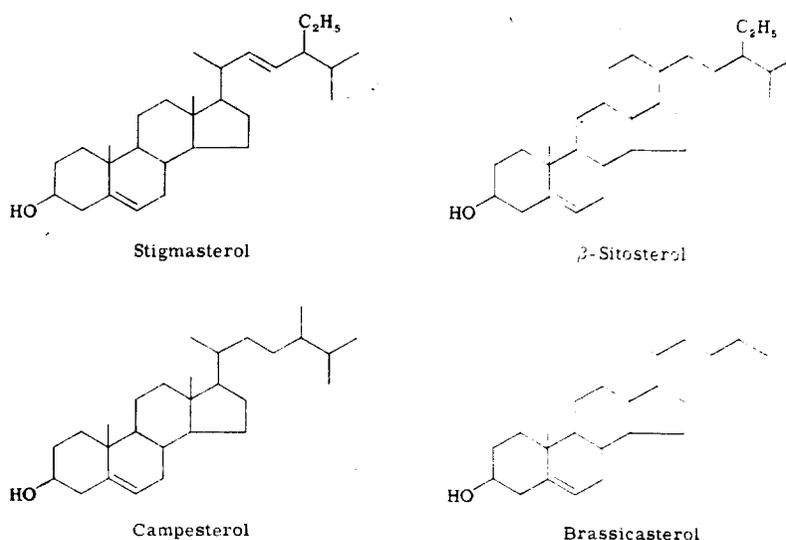
The knowledge now available concerning this group of NGC is surely destined to be enhanced in the coming years.

e. Sterols. Table VIII reports the most common members of this best known NGC group. In vegetable oils, as in all the lipids of living organs, sterols are present in both free and esterified form; as for cyclic terpene alcohols, the acids may be those of the oil itself, or ferulic acid (Tamura *et al.*, 1959, 1962b).

The sterols contained in vegetable oils are not numerous; the structures of most of them are definitely known (Table IX). A few uncertainties derive from the fact that not all the sterols which are GLC-separable have yet been studied individually; the doubts existing on some of the structures (e.g., α_1 , β_2 , γ_2 sitosterol) (Tamura *et al.*, 1959) are precedent to the introduction of GLC.

A systematic investigation of vegetable oil sterols (Capella *et al.*, 1964b; Fedeli *et al.*, 1966b; Rutkowski *et al.*, 1966) has shown that the oils always contain at least three sterols, seldom four; β -sitosterol and campesterol are always present, the former being the predomi-

Table VIII



nant constituent. Prior to the advent of GLC, β -sitosterol was considered in many cases to be the only sterol present; the difference of one oil from another, when their sterols are alike, lies in their different relative quantity.

Among other sterols revealed in the past, are fucosterol in the *Asphodelus fistulosus* oil (Khan *et al.*, 1961) and spinasterol in spinach oil (Larsen, 1938). Palm oil contains, in addition to campesterol, stigmasterol and β -sitosterol, a fourth component whose retention time in GLC is very close to that of cholesterol (Reçourt and Beerthuis, 1963). Cholesterol seems also to be a minor sterol of peanut oil (Walbecq, 1966). Definitive proofs of its identity are still lacking.

f. Tocopherols. For this group, also, the application of GLC under given conditions has permitted considerable advances. Tocopherols can be separated on columns or by TLC, starting from nonsaponifiables, carried out with special precautions, or on the oil *in toto*. Owing to the poor stability of tocopherols in the presence of air, special procedures and eluents are generally used in order to permit the separation of tocopherols alone with the exclusion of the other components (Nair and Turner, 1963); sterols in particular are troublesome because retention times are very close (P. W. Wilson *et al.*,

Table IX
NUMBER OF STEROLS IN VARIOUS VEGETABLE OILS

Oil	Number of components	R _f values					
		0.58	0.60	0.68	0.80	0.87	1.00
				Brassicasterol	Campesterol	Stigmasterol	β -Sitosterol ^a
Linseed	3				•	•	•
Peanut	3				•	•	•
Olive	3				•	•	•
Rice bran	3				•	•	•
Palm kernel	4	•			•	•	•
Corn	3				•	•	•
Sesame	3				•	•	•
Citricid	3				•	•	•
Coconut	3				•	•	•
Palm	4	•			•	•	•
Rapeseed	3			•	•	•	•
Grape seed	3				•	•	•
Sunflower	4				•	•	•
Poppy seed	3				•	•	•
Castor	3				•	•	•
Tea seed	3				•	•	•
Cocoa butter	3				•	•	•
Soybean	3				•	•	•

^aR_f relative to β -sitosterol=1.00.

1962). The subsequent GLC analysis can be performed on the free compounds or, better, on their trimethylsilyl ethers (Slover *et al.*, 1967). The interest involved in these NGC, as is known, derived from the antioxidant action of free tocopherols, among which the γ -isomer, as it appears so far, is the most active or the sole active one. Tocopherol esters, where the hydroquinone structure is blocked, cannot have antioxidant ability.

The various tocopherols present in oils, can be determined by GLC with good precision, although there is some difficulty in resolving the δ - and ϵ -isomers (Slover *et al.*, 1967). Table X, derived from Herring and Drury (1963), supplies information on the tocopherol types and quantities of some of the commonest vegetable oils.

Table X
 TOCOPHEROL TYPES AND QUANTITIES IN COMMON VEGETABLE OILS

Source of oil	Total tocopherols (mg/gm)	α Tocopherol (mg/gm)	Others present
Castor bean	291	0	γ , or β , δ
Coconut	11-24	5-16	γ , η
Corn	427-661	194-157	γ , δ
Cottonseed	298-635	230-375	γ
Grape seed	194	149	—
Linseed	61-428	0	γ , β
Olive	46-146	31-124	γ
Peanut	215	130	γ
Rapeseed	433	170	γ
Rice bran	444	264	γ , or β , η
Safflower seed	248-492	226-426	γ
Soybean	797	175	γ , δ
Sunflower seed	271	224	γ
Tung	810	243	γ , β or β
Wheat germ	1897	1276	β

It is relevant to point out here that the oxidometric titration of total tocopherols in an oil, as it is generally performed according to Emmerie-Engel (Emmerie and Engel, 1939), is not specific, but rather assays all the reducing substances that may be present (Herting and Drury, 1963).

3. Components Obtained Directly from the Oil

We have seen that the information obtainable from unsaponifiables is always incomplete and subject to some reservations; therefore, many researches have been proposed to bypass saponification.

The relative facility with which single NCG have been derived directly from an oil is an attribute, in addition to their stability, of their acidic or phenolic character which facilitated their extraction or insolubilization in nonpolar solvents; for example, compounds related to sesamol which is found in plants of the sesamum family (*Indicum angolense*) and in the bark of various *Fagara* and other species. For formulas and characteristics, we refer to the excellent review by Budowski (1964). This group of compounds can be extracted from the oil by means of alkali (Suarez *et al.*, 1952) or directly by column adsorption, by paper partition, or by TLC.

The gossypol group, the pigments of glanded cottonseed, has been extensively studied; we refer the reader to the review of Adams

(Adams *et al.*, 1960). More recent reports are also available (El-Nock-rashy *et al.*, 1963; F. H. Smith, 1967).

The presence of oleanolic acid (a triterpenic acid, of the α -amyrin series), as a consistent component of the olive fruit and of its oil, has long been known (Canzoneri, 1906; Parisi and De Vito, 1931; Scurti and Tomasi, 1912). More recent is the identification of maslinic (Caglioti, 1961) and crategolic (Vioque *et al.*, 1961) acids. The presence has yet to be confirmed in vegetable oils—in particular, olive oil—of other terpenic acids such as ursolic and betulinic (Mazuelos Vela, 1964; Thiers *et al.*, 1959); these acids are very probably in free form in the oil.

Other groups of substances have been noted, but further investigation is needed to ascertain their identification; among the surest ones, we would mention plastoquinones with a structure close to that of vitamin K₁, detected in chloroplasts (Kegel, 1962) and in olive oil (Therrault, 1963); a group of phenolic substances, whose presence has been observed in at least two oils [in parsley oil, the "myristicin" (Privett *et al.*, 1963), and in olive oil, the "oleoeuropein" (Vazquez Romero *et al.*, 1961)] and the vinylic esters and esters of diols (propane-1,2- and 1,3-diol, butane-1,3- and 1,4-diol); these last are found in corn seeds and consequently in the oil also (Bergelson *et al.*, 1966).

It is possible, however, within certain limits at least, depending on the unsaturation degree of the oil, to obtain from it by mere solvent crystallization (i.e., acetone, ethyl acetate) the oil's NGC fraction, thus avoiding saponification (Jacini and Fedeli, 1967). Obviously, it is possible to apply to the thus "enriched" fraction the aforementioned separation methods already employed with tissue lipids. By such techniques, it has recently been possible to evaluate the amount of terpene alcohols and of sterols present in the free or esterified form, bringing into evidence some points which have previously not been noticed. In olive oil, for instance, the fatty acids present in terpene alcohol and sterol esters are remarkably different from those of the oil itself, because of the presence of more than 90% of a C₂₀ acid practically absent in the glycerides of the oil itself. In olive oil the presence has been observed of a chlorophyll different from the *a* and *b* and probably identical to that isolated from chlorobium (Stanier and Smith, 1960; Jacini and Fedeli, 1967).

IV. Conclusions

It is clear from the number and quality of the works published in recent years that the composition of vegetable oils is a subject of

considerable interest, about which much remains to be learned. We may anticipate that the combined application of instrumental micro-methods will lead us to the elucidation of the relationships between the oil components and those of the photosynthetic tissues of the corresponding vegetable species, the oil biogenesis and the destiny of glycerides and of minor constituents during seed growth and germination, respectively.

In contrast, it is not easy to anticipate the practical effects of the whole complex of this research; it is hoped that better criteria may result for the selection and cultivation of oil-bearing seeds required for food purposes, the utilization of new botanic species and, finally, substantial improvements in the application of industrial techniques for extraction, refining, and preservation of vegetable oils.

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10
**Cutaneous and Parenteral Studies with Vehicles Containing
Isopropyl Myristate and Peanut Oil**

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Cutaneous and Parenteral Studies with Vehicles Containing Isopropyl Myristate and Peanut Oil. FITZGERALD, J. E., KURTZ, S. M., SCHARDEIN, J. L., and KAUMP, D. H. (1968). *Toxicol. Appl. Pharmacol.* 13, 448-453. Daily cutaneous application of isopropyl myristate induced a prompt skin response in mice and rabbits. This was characterized at first by erythema, and later by lichenification, and fissure formation. Histologically, acanthosis, para- and hyperkeratosis, focal erosion, and focal hemorrhage were seen. In rabbits, the skin lesions regressed slowly after cessation of treatment, while in mice the lesions tended to regress during continued treatment. Similar reactions occurred with combinations of isopropyl myristate and peanut oil, but the intensities of the dermatoses were generally related to the proportion of isopropyl myristate in the mixture. Peanut oil alone produced only mild gross and microscopic changes.

A mixture of 25% isopropyl myristate and 75% peanut oil produced only minor local damage without definitive systemic effects when injected repeatedly in rats, dogs, and monkeys, or when given as single intramuscular injections to rabbits.

Isopropyl myristate is a mixture composed principally of the isopropyl ester of myristic acid with lesser amounts of the isopropyl esters of other fatty acids. It is used in cosmetics and pharmaceuticals for its emollient and dispersing properties. It has been studied in our laboratory in a variety of toxicologic contexts over the past several years under the name Delyl Extra. The intraperitoneal and subcutaneous LD₅₀'s exceeded 79.5 ml/kg in rats and the intraperitoneal LD₅₀ exceeded 50.2 ml/kg in mice. Single oral doses of Delyl Extra of 16 ml/kg are tolerated by rats (The Givaudanian, 1953). Single topical applications to rabbits did not induce skin reactions, but daily repeated amounts resulted in local irritation (The Givaudanian, 1953). Limited intraperitoneal studies in mice and rats, and intramuscular irritation experiments in rats with isopropyl myristate did not reveal any specific toxic effects (Platcow and Voss, 1954). The present study was designed to obtain information on local and systemic tolerance of a parenterally administered combination of isopropyl myristate and peanut oil, and to study the topical irritation potential of each compound alone and in combination.

METHODS

Intramuscular tolerance studies. Groups of 24 male and 24 female Holtzman source rats, 4 beagle dogs, and 2 rhesus monkeys were given up to 12 weekly injections of a

25% isopropyl myristate-75% peanut oil mixture intramuscularly at levels of 0.3 ml/kg (rats), 0.14 ml/kg (dogs) and 0.15-0.33 ml/kg (monkeys). In addition to the observation and recording of grossly evident clinical reactions and weight changes, routine peripheral blood and bone marrow examinations and blood biochemical determinations were done prior to, during, and at termination of the test period. Some animals were killed at the end of the treatment period, and others at intervals up to 4 weeks after the last dose. Complete gross and microscopic examinations were conducted on all animals.

Intramuscular irritation studies in rabbits. A group of 4 New Zealand white rabbits received a single intramuscular injection of 0.5 ml of a mixture containing 25% isopropyl myristate-75% peanut oil. One rabbit was killed at 1, 7, 14, and 28 days after injection. The injection sites were removed and examined for gross and microscopic changes.

Topical irritation studies in mice and rabbits. Groups of 12 male albino Spartan source mice were tested for topical irritation with each of the following preparations: isopropyl myristate, a mixture of 75% isopropyl myristate and 25% peanut oil, a mixture of 25% isopropyl myristate and 75% peanut oil, and peanut oil. An area (approximately 1.5 inches square) on the backs of the animals was clipped prior to application and at weekly intervals during the test period. A film of each preparation was applied daily with a cotton topical applicator for periods up to 28 days, and gross observations were recorded. Two animals in each group were killed after 1, 2, 3, or 4 weeks of exposure, and the application sites were removed and processed for histologic evaluation. Half the remaining animals in each group were killed at 1 week, and the other half at 2 weeks after the last exposure. The application sites were removed and examined microscopically.

Four New Zealand white rabbits were clipped and four 2 × 3 inch areas were marked on their backs. A film of each of the above-listed preparations was applied to one of the areas with a cotton swab twice daily for 1-2 weeks. Single animals were killed after 1 and 2 weeks of exposure and at 1 and 2 weeks after cessation of exposure. At necropsy, the application sites were removed and examined grossly and microscopically.

RESULTS

Intramuscular Tolerance Studies. The weekly intramuscular injection of a mixture of 25% isopropyl myristate and 75% peanut oil did not induce local or systemic clinical signs of intolerance in rats, dogs, or monkeys. There was a slight (3-4%) reduction in food intake and weight gain in exposed rats during the treatment and reversibility periods. There were in all species slight, usually irregular, aberrations in clinical laboratory values that were not clearly related to treatment. At autopsy, there were no significant gross pathologic changes; in all species, the injection sites were inconspicuous. Microscopic examination of injection sites in rats disclosed only small focal areas of empty vacuolar structures in the muscle without associated inflammation or fibrosis.

In the monkeys, there were small focal lesions (1 week after the last dose) which consisted of small cystic spaces surrounded by macrophages. There was a slight leukocytic infiltration, and occasional small multinucleated cells in some areas. In

dogs, the injection sites contained minimal foci of residual fibrosis, and an occasional small granuloma.

Intramuscular Irritation Studies in Rabbits. The intramuscular administration of the 25% isopropyl myristate and 75% peanut oil mixture in rabbits resulted in slight edema, necrosis, and neutrophil infiltration 24 hours after injection. Seven days after injection, there was slight edema and necrosis of muscle fibers. By day 14, there was delicate encapsulation of small droplets of vehicle. Foci of residual fibrosis and a few encapsulated droplets of vehicle were observed on day 28.

Topical Irritation Studies in Rabbits and Mice. Rabbits developed a slight erythema after the first application of isopropyl myristate. After 4 exposures, the sites of application were moderately erythemic and slightly thickened. These changes progressed until there was marked thickening of the site and development of hemorrhagic areas by day 5. The lesions persisted throughout the remainder of the 14-day treatment period. Similar changes occurred with the 75% isopropyl myristate-25% peanut oil mixture but developed 2-3 days later. The 25% isopropyl myristate-75% peanut oil mixture and peanut oil alone induced a slight erythema after 5-9 days of treatment. All lesions regressed after cessation of dosing, but erythema was still evident 2 weeks after treatment with the higher concentrations of isopropyl myristate.

Microscopically, the fully developed lesion induced with isopropyl myristate was characterized by acanthosis, hyperkeratosis, parakeratosis, and dilatation and plugging of hair follicles. In some sections there were also focal erosions, congestion, hemorrhages, and edema and patchy infiltration of the dermis by granulocytes and mononuclear cells. While similar changes occurred after application of 75% isopropyl myristate and 25% peanut oil, they developed more slowly. The mixture of 25% isopropyl myristate and 75% peanut oil induced even milder and more slowly developing changes than the higher concentrations of isopropyl myristate. Peanut oil alone induced mild acanthosis, hyperkeratosis with some hair follicle plugging, and a focal dermal inflammatory response. After the applications were discontinued, the skin changes regressed; however, epidermal thickening with increased exfoliation or keratosis, some follicle dilatation and plugging, and patchy lymphoid cell infiltration was still present 14 days after treatment. These changes were mild in animals treated with either peanut oil alone or with 25% isopropyl myristate-75% peanut oil, but were more intense in animals treated with higher concentrations of isopropyl myristate.

Topical applications of the same preparations on the clipped skin of mice induced a qualitatively similar, though somewhat more variable dermatosis than was seen in rabbits. Most mice receiving isopropyl myristate alone developed exfoliation of the epidermis with underlying diffuse small hemorrhagic areas after 6 days of treatment. The lesion progressed with continued application, to thickening and keratinization of the skin by day 13. The affected areas began to slough from days 14 to 17, revealing apparently normal skin underneath. With continued application of the compound, there was only slight exfoliation observed in some animals throughout the remainder of the 28-day exposure period. Two of the 12 animals did not develop any gross signs of irritation during the treatment period, although one of the animals had slight exfoliation at the end of the reversibility period. The mixture of 75% isopropyl myristate and 25% peanut oil also induced marked dermal reactions but they developed more slowly than with isopropyl myristate alone and did not begin to regress until

after the cessation of dosing. The mixture of 25% isopropyl myristate and 75% peanut oil induced the same gross pattern of dermal reaction but the time of onset was slower and resolution more rapid than with isopropyl myristate alone. Peanut oil alone resulted in retardation of hair growth and slight to moderate exfoliation of the epidermis by day 14. These changes also regressed with continued application.

Histologically, the lesion produced in mice after 7 days of treatment with isopropyl myristate was characterized by hyperkeratosis, parakeratosis, acanthosis, keratin plugging of hair follicles and dermal infiltration by granulocytes. Similar, but somewhat less intense changes were seen after 14 days of treatment (Fig. 1). These changes diminished in prominence with continued treatment (Fig. 3). Essentially, the same reactions with the addition of occasional microabscesses and focal epidermal erosions occurred after application of the mixture of 75% isopropyl myristate and 25% peanut oil. These changes, which developed more slowly than when isopropyl myristate alone was applied, did not regress during treatment but did subside after cessation of dosing. Mice receiving either the 25% isopropyl myristate-75% peanut oil mixture or peanut oil alone developed mild to moderate hyperkeratosis, acanthosis, and parakeratosis with neutrophil and mononuclear cell infiltration of the dermis after 14 days of treatment (Fig. 2). Again these changes tended to regress with continued treatment (Fig. 4). At the end of the reversibility period, all treatment groups were essentially normal (Figs. 5 and 6), except for focal epidermal hyperplasia, focal epidermal erosions, and neutrophil infiltration in the dermis of 1 of 2 animals which had received isopropyl myristate alone.

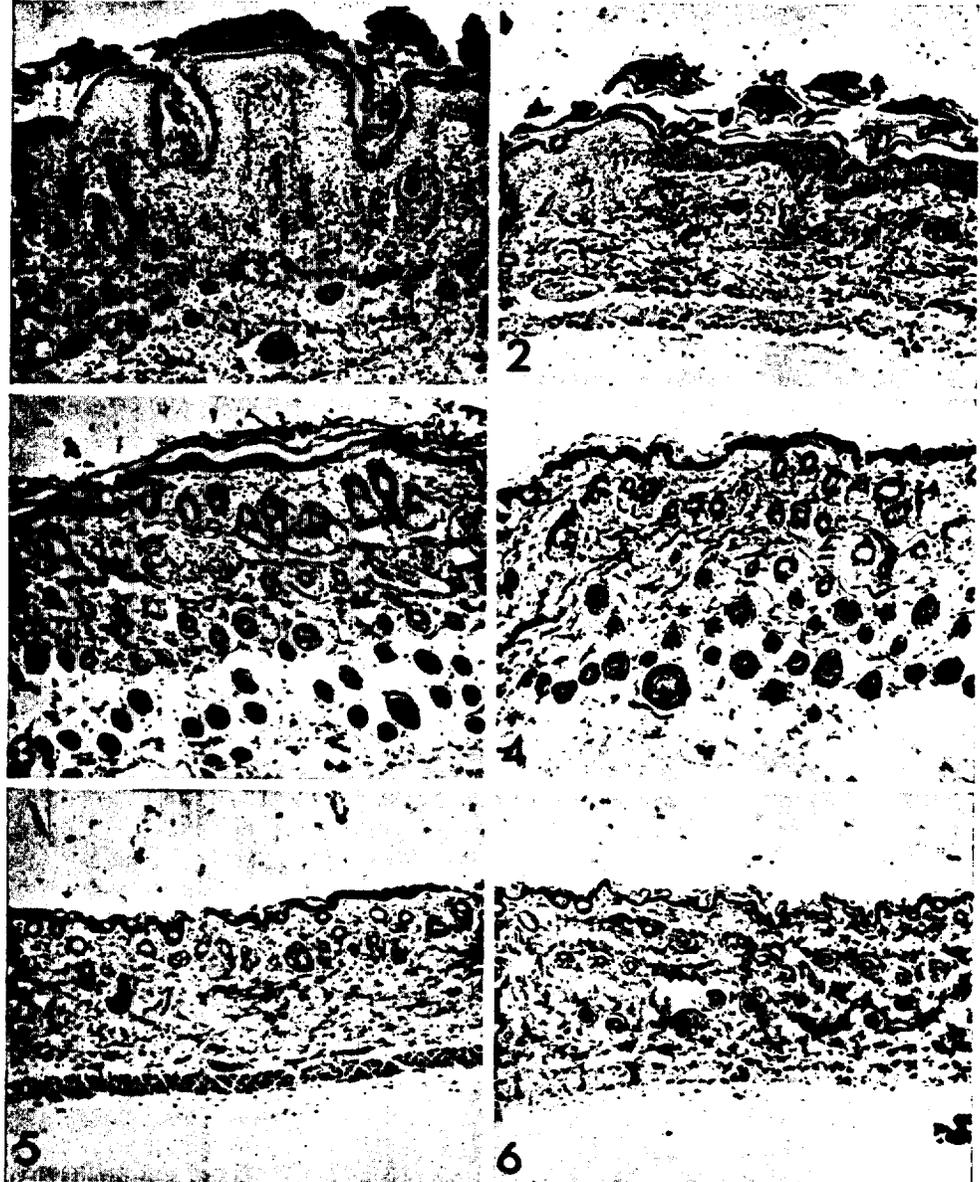
DISCUSSION

These experiments indicate that, in the dosages employed, the mixture of 25% isopropyl myristate-75% peanut oil had a low order of local or systemic toxicity when administered intramuscularly to rats, dogs, monkeys, and rabbits.

When isopropyl myristate alone or in combination with peanut oil was applied daily to the clipped skin of rabbits for 1 or 2 weeks, a mild to marked local irritation appeared. The degree of irritation generally appeared to be proportional to the relative amount of isopropyl myristate in the applications. The skin lesions regressed only after the cessation of application of the compounds.

When isopropyl myristate alone, peanut oil alone or their combination was applied topically to mice, similar skin reactions appeared. There was a distinct tendency for skin lesions to regress during continued application of the various compounds. This regression of inflammation during continuing application may not reflect real differences in reactivity of mice to the compounds, but rather may be related to the longer duration of exposure in mice.

Platcow and Voss (1954) reported a low order of toxicity when isopropyl myristate alone was administered intraperitoneally to mice or intramuscularly to rats. In their experiments, daily intraperitoneal administration of isopropyl myristate to rats for 20 days resulted in questionable toxicity; 3 of 8 animals died by day 5, while the surviving rats had no signs of toxic effects. In another study, no local irritation was observed after single applications of isopropyl myristate to the clipped skins of rabbits. However, 6 daily topical applications to rabbits resulted in severe skin irritation characterized by erythema and edema (The Givaudanian, 1953). These changes



All figures are photomicrographs of paraffin-embedded, hematoxylin-eosin stained sections of mouse skin. All figures are at a magnification of $\times 55$.

FIG. 1. Topical treatment with isopropyl myristate for 14 days. There is acanthosis, hyper- and parakeratosis, and mild dermal inflammation.

FIG. 2. Topical treatment with peanut oil for 14 days. There is mild hyperkeratosis, acanthosis, and patchy dermal inflammation.

FIG. 3. Topical treatment with isopropyl myristate for 28 days. There is slight exfoliation and minimal acanthosis.

FIG. 4. Topical treatment with peanut oil for 28 days. Minor thickening of epidermis and very slight exfoliation are present.

FIG. 5. Seven days after cessation of treatment with isopropyl myristate. The skin is essentially normal.

FIG. 6. Seven days after cessation of treatment with peanut oil. The skin is essentially normal.

progressed with continued application until the skin became "hard and leathery" after 20 days. The exposed areas sloughed by day 5 after cessation of dosing, leaving the surface dry and scaly.

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HAIR LOSS FROM SEBUM

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IN PREVIOUS publications my colleagues and I have described a group of unsaturated compounds which cause temporary loss of hair in laboratory animals, with disappearance of the hair shafts and of most of the follicles, marked acanthosis, and anomalies in keratinization. The new depilatory compounds included the synthetic dimers (intermediary polymers) of chloroprene,¹ allyl laurate, allyl benzoate, allyl diphenyl acetate, and the naturally occurring vitamin A² and squalene.³ With the exception of vitamin A, all these substances inhibited the free sulfhydryl groups of tissue proteins. We ascribed the depilatory effect to the $-C=C-$ groups in the molecules which combine with free sulfhydryl groups. Two types of representatives of these compounds, namely the chloroprene dimers⁴ and vitamin A⁵ previously have been shown to cause loss of hair in human subjects as well.

From the Department of Dermatology and Syphilology, University of Pennsylvania (Donald M. Pillsbury, M.D., Director). This investigation has been made under a Damon Runyon Senior Clinical Fellowship of the American Cancer Society, Inc., and with the assistance of a grant from the Committee on Research, Council on Pharmacy and Chemistry, American Medical Association. Part of this essay was awarded Honorable Mention in the Second Annual Essay Contest of the American Dermatological Association.

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Among these compounds squalene occupies a special position. Squalene, an unsaturated hydrocarbon, is a normal component of human sebum.⁶ Therefore the possibility was considered that human sebum itself may influence hair growth. This assumption was supported when it was found that human sebum (fat obtained by ether extraction of hair collected in barber shops) caused complete local depilation in rabbits and in mice after a single application.⁷ In vitro, human sebum, like the other depilatory agents, inhibited sulfhydryl compounds and the sulfhydryl enzyme, succinic dehydrogenase.¹¹

However, certain observations remained unexplained. In the first place, even repeated applications of squalene failed to produce baldness in mice, while a single local treatment with human sebum gave rise to loss of hair in a high percentage of these animals. Possibly even of greater importance was our finding that the sulfhydryl-inhibitory potency of human sebum was several hundred times greater than could be accounted for on the basis of its reported squalene content.

Summarization of *In Vivo* Studies

Substance Tested	Rabbits		Guinea Pigs		Mice	
	Experiments, No.	Amounts Used, Ml.	Experiments, No.	Amounts Used, Ml.	Experiments, No.	Amounts Used, Ml.
Human "sebum" (hair fat).....	14	1 to 4	4	1	23	0.1 to 0.3
Unsapoifiable fraction	3	0.5
Sapoifiable fraction (total fatty acids)	3	0.5
Squalene *	18	1 to 4	10	1	26	0.3
Oleic acid U.S.P. (Merck).....	5	1 to 2	2	1	12	0.3
Aerated oleic acid f.....	3	1 to 2	2	1	10	0.3
Linoleic acid (Eimer and Amend).....	5	1 to 2	10	0.3

* Two types of squalene were used: (1) "technical grade" from Distillation Products Industries, Rochester, N. Y.; (2) a highly purified preparation, obtained through courtesy of Alimentation Equilibree, Commeny (Allier), France.

† Oleic acid was aerated at a slow rate for one week at room temperature. The purpose of this experiment was to test whether or not formation of peroxides under aeration enhanced the depilatory power of this compound.

Therefore we concluded that the depilatory action of human sebum could not be due solely or even chiefly to its squalene content. In addition to squalene, sebum appeared to contain other, more potent, depilatory compounds.

The present report discusses the identification of some of the depilatory compounds of human sebum. Experiments were carried out to test various fractions and components of human sebum for their depilatory effect upon laboratory animals and for their sulfhydryl-inhibitory activity in tissues.

MATERIAL AND METHOD OF STUDY

I. *In Vivo* Studies with Human Sebum and its Components.—"Sebum" was obtained by extracting with ether hair collected in barber shops, filtering the extract, and evaporating the ether. The hair was derived from male adults and from children and was separated into prepubertal and postpubertal samples. In a few experiments acetone or chloroform was used to

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7. Flesch and Goldstone.¹¹ Flesch and Hunt.

extract hair. Fractionation of the sebum into saponifiable (total fatty acids) and unsaponifiable parts was performed by the method of the Society of Public Analysts.⁸

Animal experiments were carried out by painting human sebum, its fractions, and the individual components on the skin of rabbits, guinea pigs, and mice. Animals of both sexes and all age groups were used. All substances tested were applied only once from pipettes. These substances then were rubbed with a glass rod into the skin directly in front of the ears for 20 seconds. To ensure its proper spreading and distribution, sebum was warmed to body temperature. We had available for *in vivo* studies only small amounts of the fractions of sebum; therefore, in order to facilitate contact with the skin, the animals' hair was clipped before application of these fractions. The *in vivo* studies are summarized in the accompanying table.

A control series of animals was treated with stearic acid (25 to 50% alcoholic solution), liquid petrolatum, and saturated ethereal solutions of hydrous wool fat.

Biopsy specimens were taken 10 days after treatment from the depilated and from the control series. Routine histologic sections were prepared and stained with hematoxylin and eosin.

2. *In Vitro* Studies.—Free sulphydryl groups were determined in tissue homogenates and in glutathione after incubation with sebum and its derivatives. One-tenth milliliter of 10% aqueous mouse liver homogenate, 20% human epidermal homogenate,⁹ or 0.1 ml. of aqueous glutathione solutions (containing 40 to 60 gm. glutathione) was buffered with barbiturate buffer to pH 7.4. These mixtures were incubated with 0.1 ml. of 100% alcoholic suspensions of human sebum and of its fractions or with alcoholic solutions of oleic and linoleic acids. Squalene was used in ethereal solutions. The dilutions ranged from 1:2 to 1:500. Control tubes contained the solvents in place of sebum and its derivatives. The incubation was carried out at room temperature for 20 minutes or in a water bath at 36 C for 10 minutes. At the end of this period the free sulphydryl groups were determined with Bennett's reagent.¹⁰

The activity of a sulphydryl enzyme, succinic dehydrogenase, was measured with the method of Kun and Abood.¹¹ One milliliter of 10% aqueous mouse liver homogenate, mixed with barbiturate buffer of pH 7.4, was incubated with sebum and its derivatives in the same way as described above. Control tubes contained the solvents only.

Since sebum obtained from hair collected in barber shops was invariably contaminated with extraneous material (hair lotions, etc.), it was used only for preliminary studies. Further *in vitro* experiments were carried out with purer samples of sebum, collected in the Kettering Laboratory of the University of Cincinnati.¹¹ The contents of individual sebaceous glands on the nose, chin and cheeks were expressed; the sebum was dried over CaCl₂ under reduced pressure and sent to our laboratory in sealed tubes, marked only with numbers. The weights of these samples ranged from 1 to 4 mg. The sebum was dissolved in ether, quantitatively transferred to centrifuge tubes, and the solvent evaporated. The dried sebum was suspended in alcohol in a final concentration of 1:50 to 1:100, and these suspensions were used for studies of sulphydryl inactivation and succinic dehydrogenase-inhibition.

RESULTS

1. *In Vivo* Studies.—A single application of human sebum caused reversible loss of hair in all rabbits (Fig. 1) and in 10 of the 23 mice tested (Fig. 2). No hair loss was observed in guinea pigs. Crusting and thickening of the skin developed four to five days after treatment. The first signs of hair loss appeared on the seventh day after application. The hair was cast off in the form of tufts with adherent scales.

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The underlying skin emerged smooth and completely hairless. Regrowth of hair began a week later, and within a few weeks the fur had resumed its normal appearance.

Both the unsaponifiable and the saponifiable fractions caused loss of hair in all rabbits tested. The sequence of events closely resembled that observed with whole sebum.

Oleic and linoleic acids were very potent depilatory agents in all species tested. No appreciable difference could be observed between the depilatory potencies of aerated and nonaerated oleic acids. Both unsaturated acids had a marked irritating effect on the animal skin, causing crusting, ulceration, and thickening. However, all these changes were reversible. The depilatory action of squalene has been described previously. The present work, utilizing the highly purified product of Alimentation Equilibree, confirmed earlier results obtained with less pure ("technical grade") preparations.



FIG. 1.—Hair loss in rabbit eight days after single application of human sebum.

The histologic findings at the height of the hair loss were essentially the same in all animal species and with all the depilatory compounds tested. The most important changes were hyperkeratosis; pronounced acanthosis, assuming at times the proportions of pseudoepitheliomatous hyperplasia; keratotic plugs in the follicles; hyperplasia of the sebaceous glands, and disappearance of the hair shafts and of many of the hair follicles (Fig. 3). All these changes were reversible.

In the control series, hydrous wool fat, liquid petroleum, or stearic acid had no demonstrable effect on hair growth, even after repeated applications. The histologic features of the skin after such treatment were essentially those of normal skin.

2. *In Vitro* Studies.—*In vitro*, sebum (both the hair fat and the purer mixtures obtained directly from the sebaceous glands), its fractions, and its unsaturated depilatory components (activated sulfhydryl compounds and the sulfhydryl enzyme, succinic dehydrogenase). The percentage inactivation revealed a linear relationship when plotted on semilog paper against the amounts of sebum used (Figs. 4 and 5).



Fig. 2.—Hair loss in mouse 10 days after single application of human sebum.

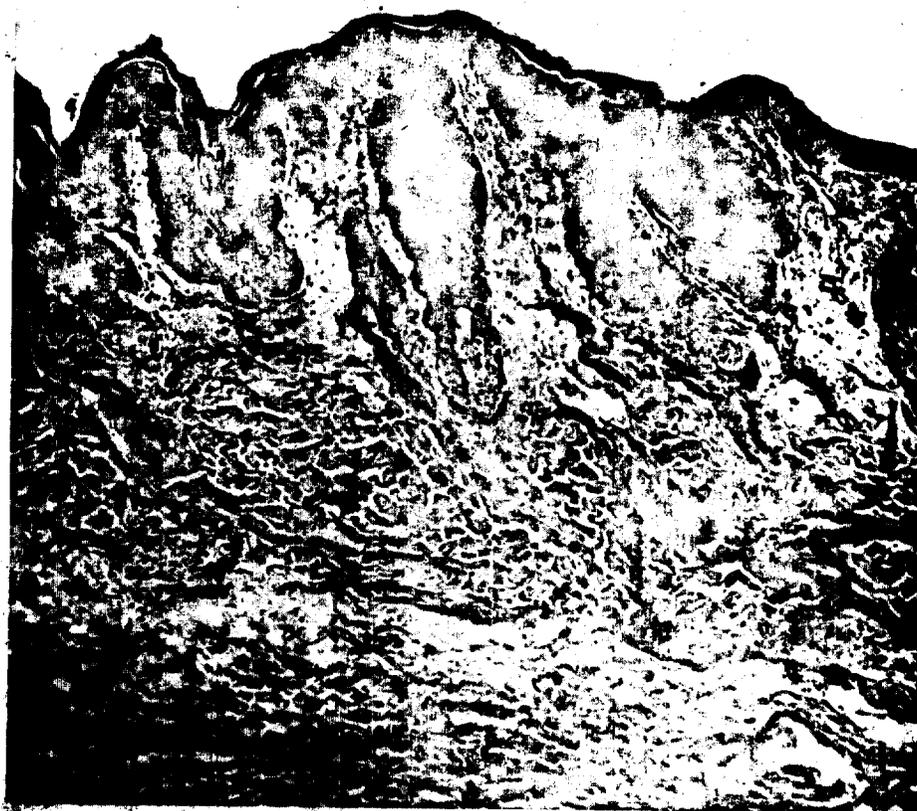


Fig. 3.—Rabbit skin 10 days after treatment with oleic acid; $\times 80$. Note keratotic plugs, excessive acanthosis, hyperplastic sebaceous glands, and absence of hair shafts.

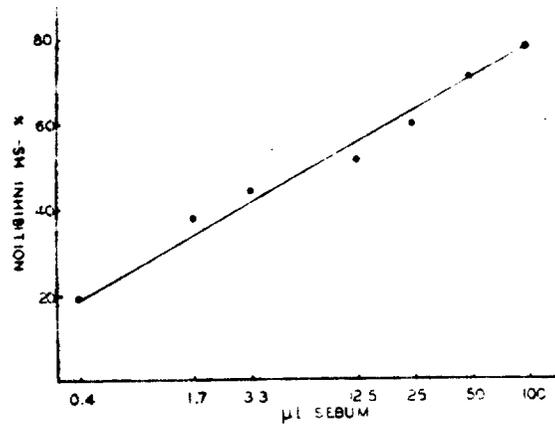


Fig. 4.—Inactivation of sulfhydryl groups in mouse liver by human sebum.

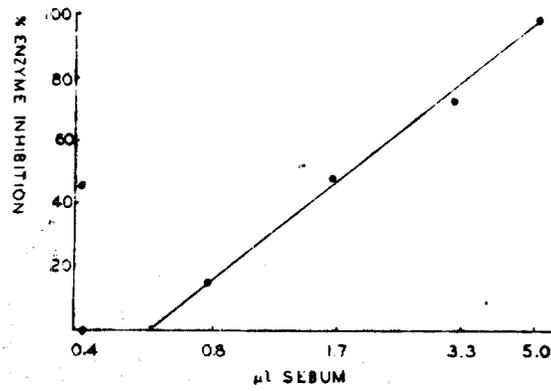


Fig. 5.—Inhibition of succinic dehydrogenase activity of mouse liver by human sebum.

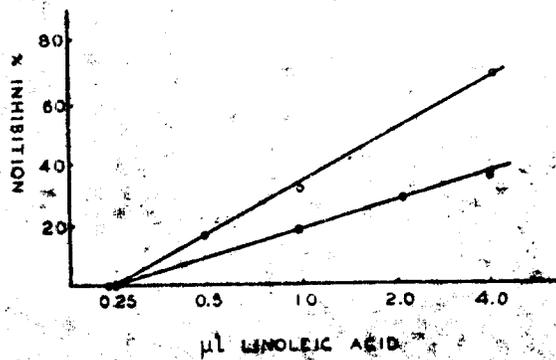


Fig. 6.—Inactivation of sulfhydryl groups and succinic dehydrogenase in mouse liver by linoleic acid. Black dots, sulfhydryl groups; white dots, succinic dehydrogenase per 100 mg. of tissue.

The sulfhydryl-inhibitory concentrations of oleic and linoleic acids are plotted in Figure 6. Samples of aerated and nonaerated oleic acids had identical sulfhydryl-inhibitory effects.

Stearic acid, having a much higher melting point than any of the other compounds tested, precipitated in the aqueous reaction mixtures from its alcoholic solutions, and therefore its *in vitro* effect could not be evaluated. Liquid petrolatum and hydrous wool fat had no demonstrable effect on glutathione or succinic dehydrogenase even in 10% solutions. However, in these high concentrations, hydrous wool fat inactivated some of the sulfhydryl groups of liver homogenates.

COMMENT

From our experiments with sebum and its derivatives, the following two conclusions may be drawn:

1. Human sebum inhibits hair growth and influences keratinization in laboratory animals in some as yet unknown manner. Although the sebum used in our experiments was contaminated with extraneous material, its three depilatory components, squalene and oleic and linoleic acids, were chemically pure and caused temporary baldness in the same way as sebum itself. In view of the fact that sebum contains a large number of other unsaturated fatty acids,¹² it seems likely that there are still other depilatory components in sebum.

2. The depilatory effect is related to the presence of $-C=C-$ groups in the depilatory compounds. Detailed evidence for this statement will be presented in a forthcoming publication.

The theory that the depilatory action is due to interference with sulfhydryl groups still rests on indirect evidence. In this respect it is noteworthy that cysteine previously has been shown to restore growth of hair in a strain of hairless rats, while cystine had no such effect.¹³ In rats made bald from a lactoflavin-deficient diet hair growth was stimulated with cysteine or glutathione.¹⁴

The presence of peroxides apparently plays a minor role in the depilatory action of oleic acid, because aeration does not enhance the depilatory or sulfhydryl-inactivating effectiveness of this compound.¹⁵ It is conceivable that commercial oleic acid contains sufficient peroxide to catalyze the reaction between the $-C=C-$ and sulfhydryl groups.¹⁶

Although the mechanism of the depilatory action of sebum and of its unsaturated derivatives is incompletely understood, a few general remarks on this type of baldness may be made even at this early stage.

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For the development of any kind of baldness, two conditions must be fulfilled: 1. The hair must be shed. 2. No new hair must replace it. The unsaturated lipid-soluble depilatory compounds all interfere with the formation of normal, new hair. This interference is manifested by the thinning of the roots of the avulsed hairs (similar to that seen in alopecia areata and temporary x-ray epilation), by the empty hair follicles, and by the amorphous keratinous masses which fill the seemingly still functioning follicles.^{1b}

Are these findings applicable to human beings? Does sebum interfere with hair growth and influence keratinization also in man? If it were definitely shown that sebaceous secretion affects the most important metabolic function of the epidermis—namely, keratinization—then eventually we would be compelled to revise a number of our concepts in the pathology and therapy of cutaneous disorders.

At present the question cannot be answered definitely. Supporters of such a theory may point to the following indirect evidence in its favor:

1. Since chemically unrelated, unsaturated lipid-soluble compounds (the chloroprene dimers and vitamin A) cause loss of hair in man, it is logical to assume that other unrelated unsaturated compounds, such as occur in sebum, also interfere with hair growth.

2. The *in vitro* effective sulphydryl-inactivating concentrations of sebum are in the same range as the amounts encountered on the skin.¹⁷

3. Male sex hormones are essential for the development of common male baldness¹⁸; they are also powerful stimulants of sebaceous secretion.¹⁹

Against the theory that sebum or its components interfere with hair growth in man, the objection may be raised that in clinical studies squalene, oleic acid, and linoleic acids had no depilatory effect. However, these experiments were conducted on a small scale and over relatively short periods. It is also conceivable that the lack of effectiveness of these compounds in human subjects is due to the longer hair cycle in man, as compared with animals. Since these compounds act on growing hair only, their effect on human scalp hair, which has a life span of several months to years,²⁰

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18. (a) Hamilton, J. B.: Male Hormone Stimulation is Prerequisite and Incitant in Common Baldness, *Am. J. Anat.* **71**:451 (Nov.) 1942; (b) Patterned Loss of Hair in Man: Types and Incidence, *Ann. New York Acad. Sc.* **53**:708 (March) 1951.

19. Hamilton,^{18a} Rony, H. R., and Zakon, S. J.: Effect of Androgen on Sebaceous Glands of Human Skin, *Arch. Dermat. & Syph.* **48**:601 (Dec.) 1943. Hamilton, J. B.: Male Hormone Substance: Prime Factor in Acne, *J. Clin. Endocrinol.* **1**:570 (July) 1941. Ebling, F. J.: Sebaceous Glands: Effect of Sex Hormones on Sebaceous Glands of Female Albino Rat, *ibid.* **5**:297 (June) 1943. Rothman, S.; Haskin, D., and Lasher, N.: Unpublished experiments.

20. (a) Pinkus, F.: Die normale Anatomie der Haut, in Jadassohn, J., *Handbuch der Haut- und Geschlechtskrankheiten*, Berlin, Julius Springer, 1927, Vol. 1. (b) Myers, R. I., and Hamilton, J. B.: Regeneration and Rate of Growth of Hairs in Man, *Ann. New York Acad.*

may not become noticeable except after prolonged exposure. This assumption is supported by a case of hypervitaminosis A in an adult, described by Sulzberger and Lazar.²⁶ In this patient the eyebrows and eyelashes, which are known to have a short life cycle,^{20a} had almost completely disappeared at a time when the hair on the scalp, though considerably thinned, was still present.

In summary, it may be stated that the evidence for the influence of sebum on human hair growth and keratinization must be considered indirect and incomplete. Further experiments with various samples of sebum and with new experimental procedures are required before this theory can be considered as definitely established. Experiments of this nature are in progress.

In the meantime the unsaturated local depilatory compounds have gained a definite position in investigative dermatology. The depilatory action of squalene, oleic acid, and linoleic acid has been confirmed and extended by independent observers. These compounds are currently used in studies on cutaneous virus infections,²² cutaneous allergic manifestations in animals,²³ studies with vesicant agents,²⁴ and protective ointments.²⁵ By converting the thin hairy animal epidermis to a thick hairless type, resembling human skin, these compounds made animal skin available for new studies. As in any other recent field, the new questions raised outnumber those which can be answered at present. Nevertheless, the introduction of these compounds into the field of investigative dermatology opens new approaches for studies of hair growth and for the development of new depilatory agents.

SUMMARY

Human sebum (obtained by ether extraction of hair), its unsaponifiable and saponifiable fractions, and three of its unsaturated components—squalene and oleic, and linoleic acids—were found to be powerful local depilatory agents in laboratory animals after only one application to the skin. The hair loss began 10 to 12 days after treatment and was always reversible. A control series of animals treated with liquid petrolatum, hydrous wool fat, or stearic acid showed no impairment in hair growth.

The main histologic changes in the depilated skin were marked acanthosis, disappearance of the hair shafts and of many of the hair follicles, and anomalous keratinization. All these changes were reversible.

In vitro, sebum and its depilatory components inactivated the free sulfhydryl groups of glutathione and of tissue homogenates and the sulfhydryl enzyme, succinic dehydrogenase.

The possibility is discussed that sebum may influence hair growth and keratinization in man.

Dr. Fred D. Weidman performed the histologic studies. Dr. Donald M. Pillsbury and Dr. R. L. Mayer gave advice and encouragement during the study. Dr. Raymond R. Suskind supplied samples of pure sebum; Dr. J. Matet, of Alimentation Equilibree, samples of squalene and perhydrosqualene, and Abbott Laboratories, synthetic vitamin A palmitate.

21. Footnote deleted.

22. Blank, H.; Personal communication to the author.

23. Suskind, R. R.; Personal communication to the author.

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STUDIES ON THE MODE OF ACTION OF VITAMIN A*

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Although there is a voluminous literature on the use of large doses of vitamin A in the treatment of diseases characterized by excessive or abnormal keratinization (e.g. Darier's disease, pityriasis rubra pilaris, ichthyosis, calluses, keratodermas, etc.), nothing definite is known about the mode of action of this vitamin in cutaneous metabolism. It has been shown that vitamin A has an "anti-keratinizing" effect which is manifested in a number of ways: *In vitro* vitamin A prevents the differentiation of epidermal cells into keratin in tissue cultures (1). In animals, large doses of vitamin A given by mouth (2), or applied locally (3), interfere with hair formation and cause reversible baldness. A decreased rate of keratin formation was also suggested by observations made on rats after local application of the vitamin (4). In human hypervitaminosis A, temporary loss of hair is a common symptom (5). Finally, vitamin A frequently has been an excellent therapeutic agent in the treatment of different types of anomalous keratinizing processes (6).

The exact mechanism of this anti-keratinizing effect is unknown. Knowledge of this mode of action may not only shed more light on the process of keratinization, but may help us to understand the role of vitamin A in the metabolism of the skin in general. Such an insight may also pave the way for the introduction into dermatology of other, possibly more potent, stable and less expensive "anti-keratinizing" compounds.

In our recent works on the depilatory effect of unsaturated lipid-soluble compounds, we have advanced a theory concerning the mode of action of large amounts of vitamin A. We suggested that in such large amounts, vitamin A had a direct, local and probably non-specific drug effect on the epidermal cells (7). The anti-keratinizing action of vitamin A has been attributed by us to the unsaturated double bonds in the molecule. We thought that vitamin A, by virtue of its unsaturation, interfered with sulfhydryl metabolism in the same way as other unsaturated, lipid-soluble depilatory agents (8).

This theory had certain shortcomings. It left unexplained the fact that after local application of vitamin A, hair loss occurred in animals only, while systemic administration of excessive doses interfered with hair growth in animals and human subjects alike. The belief that vitamin A prevented hair growth by interfering with sulfhydryl metabolism, was not supported by experimental evidence, as vitamin A palmitate had no effect on sulfhydryl compounds *in vitro*. We postulated that maybe not vitamin A itself, but some of its degradation products were responsible for the anti-keratinizing activity of vitamin A; such degradation

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products would probably also inactivate sulfhydryl compounds *in vitro*. This belief, however, was without experimental foundation (8).

Recently Wald, in his studies on visual purple, found that retinene₁, which is chemically vitamin A₁ aldehyde, the carotenoid portion of rhodopsin, combined with the free sulfhydryl groups of the protein opsin, the other component of visual pigment. Retinene₁ also combined with other sulfhydryl compounds (9). This reaction took place rather slowly. It was obvious that our own previous findings were in need of revision with the use of different derivatives of vitamin A, tested in high concentrations.

The present paper attempts to shed light on the mechanism of the anti-keratinizing action of large doses of vitamin A in the following ways:

1. In order to answer the question, whether or not there was a basic difference between local and systemic depilation, we administered to laboratory animals an unsaturated compound by mouth. This compound, oleic acid, has been described by us (8, 10), as well as by others (11) as having a local depilatory effect in animals. If it were possible to show that such a depilatory effect could be obtained by oral administration as well, it could be assumed that the two effects were basically identical and that the same conditions may also apply to vitamin A.

2. In order to study the mode of action of vitamin A, we tested several of its derivatives both for their depilatory effect *in vivo* and their sulfhydryl inactivating action *in vitro* in an attempt to find a correlation between chemical structure and reactivity.

3. Finally, some of the vitamin A derivatives were subjected to clinical trial on patients with ichthyosis.

EXPERIMENTAL

1. Oral administration of oleic acid to laboratory animals

Two groups of four albino rabbits each were given oleic acid (Merck) by stomach tube. The first group received a total of 40 ml. of oleic acid, administered in 4 equal doses every other day; of the second group, 2 rabbits received 2 doses of 2.5 ml. of oleic acid and two received the same amount of ethyl oleate at 5 days' intervals. The rabbits weighed 3 to 4 kg. Great care was taken not to contaminate the skin in order to avoid the local depilatory effect of oleic acid or ethyl oleate. Control experiments could not be carried out, because stearic acid, the saturated derivative of oleic acid, is solid at room temperature and cannot be administered by mouth, unless it is dissolved in a solvent. Introduction of a solvent would have added another variable to the experimental procedure.

2. *In vitro* and *in vivo* effects of vitamin A derivatives

- a. *Methyl, phenyl ethers and palmitic ester of vitamin A.* The methyl and phenyl ethers of vitamin A* were tested for their effect upon free sulfhydryl groups of tissue proteins and upon the sulfhydryl enzyme, succinic dehydrogenase, by

* Received through the courtesy of Dr. Roger A. Lewis and Hoffmann-LaRoche, Inc.

methods described in previous publications (12, 13). Buffered mouse liver homogenates were incubated with the alcoholic solutions of the ethers at room temperature for 15 minutes and the sulfhydryl content was compared with control mixtures, containing the solvent instead of the vitamin A ethers. Similar experiments were carried out with vitamin A palmitate. The concentrations of the vitamin A ethers used were as follows: 88 to 350 μ g. of methyl or phenyl ether and 375 μ g. to 3 mg. (1250 to 10,000 units) of vitamin A palmitate. These were added to 10 mg. (for —SH determinations) and 100 mg. (for succinic dehydrogenase determinations) of mouse liver in 10% buffered homogenates.

When using Bennett's reagent for sulfhydryl determinations in the presence of these compounds or of the synthetic precursors to be discussed below, it is essential to set up control tubes containing the vitamin A derivatives in amyl acetate solution without added tissues. We found that upon acidifying with concentrated hydrochloric acid, as is required in the method, these solutions develop a more or less marked and immediate dark color, ranging from purple to olive green, depending on the compound and its concentration. This color development may give rise to false readings. Several methods may be used to overcome this difficulty: one is to make the colorimetric readings immediately after addition of the HCl, before color development can take place. This is effective with the synthetic vitamin A precursors, but fails with higher concentrations of the esters of vitamin A where the color appears immediately upon addition of HCl. In these instances no HCl was added; readings were made by comparing a blank control amyl acetate solution containing vitamin A esters only with the supernatant amyl acetate solution of Bennett's reagent obtained from the reaction mixture which contained the tissue homogenate and the vitamin A ester. Although this procedure is less sensitive than the standard method, described by us, in which HCl is added, it is a permissible and adequate method and has been successfully used by other workers (14).

b. *In vivo studies.* To two groups of 7 mice each, alcoholic solutions of the methyl and phenyl ethers of vitamin A were administered locally on the backs every other day for 10 days. The amount applied was $\frac{1}{2}$ ml. of a solution, containing 1.75 mg. of ether/ml.* Control groups of animals were treated with alcohol only. Not enough material was available for more extensive experiments.

3. Synthetic precursors of vitamin A. (*Hydroxenin and oxenin*)

Hydroxenin and oxenin† are chemical intermediates in the manufacture of synthetic vitamin A. From their chemical configuration (Fig. 1) it is readily visible that these compounds differ from the esters of vitamin A in two respects: in the distribution of the double bonds in the side chains and in having a free alcoholic group at the end of the side chain. Since the isoprene-like chemical arrangement of the double bonds in the side chain of vitamin A is essential for the vitamin activity (15), these synthetic precursors have no vitamin A activity.

The physical properties of oxenin and hydroxenin are also different from those

* This amount corresponds approximately to 5500 I.U./cc.

† Received through courtesy of Dr. Elmer L. Sevringhaus and Hoffmann-LaRoche, Inc.

of vitamin A alcohol or of its esters. Hydroxenin is a yellowish-white crystalline material which is fairly stable at room temperature. Oxenin is a yellow amorphous powder which is unstable at room temperature and colligates after standing for a short while. In solution, both substances deteriorate rapidly.

No extensive animal studies were made with these compounds. Previous experience with other unsaturated depilatory agents has taught us that hair loss cannot be achieved by local application of solid material in alcoholic solutions to the skin of laboratory animals: The alcohol evaporates, leaving a thin film of inert material on the skin surface. This observation was confirmed also in the case of oxenin and hydroxenin. Application in oily solvents was postponed, because of the scarcity of the available supplies and their use for clinical studies.

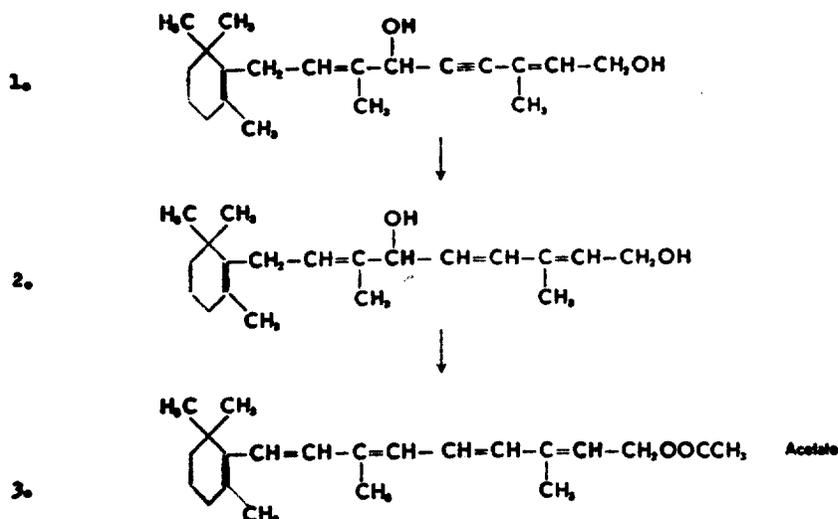


FIG. 1. Synthetic precursors in the manufacture of Vitamin A acetate. (1) Oxenin (2) Hydroxenin (3) Vitamin A Acetate.

a. In vitro studies. *In vitro* experiments concerning the effects of these compounds on sulfhydryl groups and succinic dehydrogenase were performed in the same way as described above for the ethers of vitamin A. Ten per cent mouse liver homogenates (10 mg. for —SH determination and 100 mg. for succinic dehydrogenase) were incubated with 0.1 ml. of a 0.25 to 2% alcoholic solution of oxenin or hydroxenin at room temperature for 15 minutes. The colorimetric readings were compared with control tubes containing the solvent instead of the vitamin A precursors. Readings were made both before and immediately following addition of concentrated HCl to Bennett's reagent, in order to avoid the interference of the secondary color development, as mentioned previously.

b. Clinical studies. Hydroxenin and oxenin were incorporated into cold cream bases in a 5% concentration. Sustane® (3-tertiary butyl-4-hydroxyanisole) was added in 0.02% final concentration as an antioxidant, to slow down the oxidative

deterioration of the vitamin A derivatives. The ointments were applied daily to the arms of patients with ichthyosis; the contralateral arm served as control and was treated with the cold cream base only.

Because of the limited supplies available and in order to insure properly continued treatment, only two patients could be tested with each ointment. The patients were instructed to keep the ointments stored in the refrigerator between treatments. The areas under treatment were checked every two weeks.



FIG. 2. Loss of hair on ear of rabbit after oral administration of oleic acid. Note fine scaling. The black mark above the bald area is part of an identification tag.

RESULTS

1. Oral administration of oleic acid to rabbits

Of the two groups of rabbits given oleic acid, only those receiving the higher doses developed any hair loss. One animal in this group died; the others showed no apparent ill effect except for the cutaneous symptoms to be described.

On the 5th to 7th day of the treatment, all surviving rabbits in this group developed scaling "seborrheic" lesions on the outer sides of both ears. The affected area was oval, about $1 \times 1\frac{1}{2}$ in. in diameter. The lesion was similar to the early manifestations observed after local application of oleic acid; however, the scaling was finer and there was no sign of cutaneous irritation. Shortly after the onset of the scaling the hair began to fall out with the roots. The hair loss was similar to that observed after local application of oleic acid (Fig. 2). It was reversible in all cases.

No effect was noted in any of the animals after treatment with the smaller doses of oleic acid or ethyl oleate.

2. *In vitro* and *in vivo* effects of the ethers of vitamin A

When used in sufficiently high concentrations, the methyl, phenyl ethers and palmitic ester of vitamin A inactivated the -SH groups of mouse liver homogenates and the sulfhydryl enzyme succinic dehydrogenase. The approximate minimum concentrations which still cause a significant inhibition under our experimental conditions are listed in Table I.

TABLE I
Inactivation of sulfhydryl compounds by ethers of vitamin A

	CONCENTRATIONS OF ESTERS OF VITAMIN A		
	Methyl ether	Phenyl ether	Palmitic ester
-SH groups of 10 mg liver	90 μ g	90 μ g	300 μ g
Succinic dehydrogenase activity of 100 mg. liver	175 μ g	175 μ g	1500 μ g

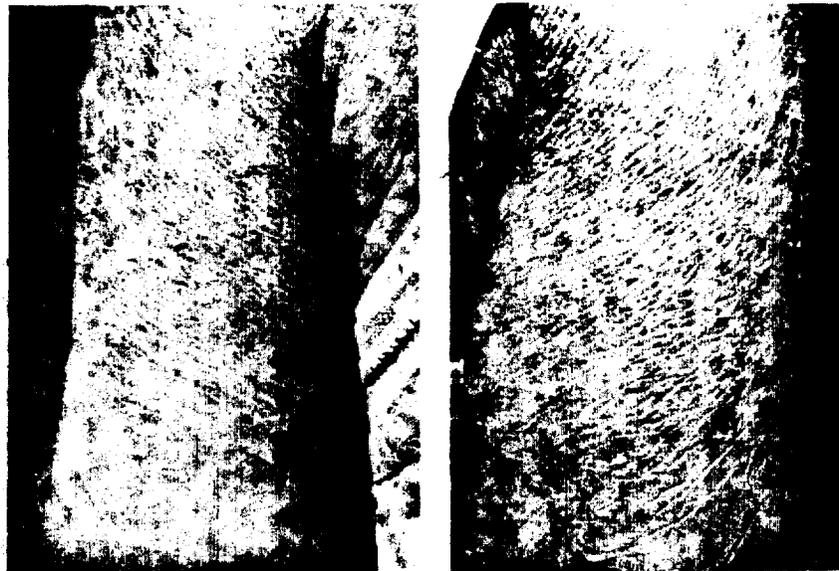


FIG. 3. Reduction of scaling in ichthyosis after 4 weeks' treatment with 5% hydroxenin ointment. Left: Control site treated with ointment base. Right: Central area treated with hydroxenin ointment.

With higher concentrations the inhibition increased until complete inactivation was reached.

Among the two groups of mice treated locally with the ethers of vitamin A, three of the animals receiving the methyl ether died within 1 week; the survivors all lost their hair on the 10th day of the treatment. No changes were observed in the group receiving the phenyl ether.

3. Effects of hydroxenin and oxenin

a. *In vitro effects.* Both synthetic precursors of vitamin A inactivated the sulfhydryl groups and succinic dehydrogenase of mouse liver. The approximate amounts necessary to cause a minimal inactivation of the sulfhydryl groups of 10 mg. mouse liver were 500 μ g. of hydroxenin and 150 μ g. of oxenin. The minimum amounts needed to inhibit the succinic dehydrogenase activity of 100 mg. of mouse liver were 500 μ g. of either compound.

b. *Clinical results.* Two to three weeks after the beginning of the treatment, both patients showed diminished scaling on the areas where hydroxenin ointment had been applied. No change was observed on the control sites treated with the ointment base (Fig. 3.). After improvement had set in, in one of the patients the hydroxenin ointment was replaced with the ointment base, whereupon the scaling returned in its former intensity. Upon resumption of the treatment with hydroxenin, the skin symptoms were alleviated again.

Inunction with oxenin ointment had no noticeable effect. Neither compound caused any local or general untoward reaction.

DISCUSSION

Cutaneous changes in rabbits after oral administration of large amounts of oleic acid have been described by Adler. With this method this author was able to produce crusted lesions on the ears of the animals. It is probable that these cutaneous changes were identical with those observed by us, although Adler failed to describe the subsequent hair loss (16). Interference with hair growth after internal as well as local administration of unsaturated depilatory compounds strongly supports the view held by us that in either case the compound (oleic acid or vitamin A) acts directly on the hair follicles. When given by mouth in large amounts, these compounds or their degradation products saturate the organism and it appears that they are partly excreted through the sebaceous glands into the hair follicles and onto the skin surface. The observations of Perutz and his coworkers strengthen this assumption. When these authors injected the highly unsaturated cod liver oil into rabbits, the hair growth decreased and the iodine number of the skin fats rose, as an indication of increased unsaturation (17).

The question why the hair loss is limited to the ears cannot be answered at present. The greater sensitivity of the hair follicles on the human scalp to a variety of physical and toxic influences is a similar unsolved problem. Preliminary histologic studies of the depilated ears of the rabbits have not yielded any satisfactory explanation.

The finding that *in vitro* the ethers of vitamin A inactivate sulfhydryl compounds is of theoretical as well as practical interest. This is the first instance in which the chemical reactivity of the ethers of vitamin A toward proteins has been demonstrated. The reaction is apparently not limited to retinene₁, the aldehyde of vitamin A₁, but is a common property of various vitamin A derivatives. Thus there is no more exception to the rule governing the depilatory action of unsaturated, lipid-soluble compounds: all these compounds inactivate sulf-

hydriyl groups *in vitro*, probably by virtue of their unsaturated groupings. Saturation (hydrogenation) abolishes the *in vivo* depilatory and *in vitro* sulfhydryl inhibitory action. The reverse, however, does not hold true: many compounds which interfere with sulfhydryl metabolism *in vitro* do not have any effect on hair growth.

This sulfhydryl inhibitory activity of vitamin A could not be demonstrated in our previous publications because the amounts of vitamin A ethers used were not sufficiently large, never exceeding 1000 units in the reaction mixtures. In the present work these amounts were increased 10 fold or more. The justifiable criticism may be raised that these amounts are unphysiologic. It should be remembered, however, that the amounts of vitamin A administered in keratinizing diseases are equally unphysiologic, surpassing the normal requirements 20 to 100 times. Even in Wald's *in vitro* studies on the reactivity of retinene₁ toward sulfhydryl compounds, 5 hours' incubation was necessary before the inactivation could be effected (9). This is a very long period for biochemical studies of this nature. In our work, 15 minutes' incubation was used routinely and it is likely that the amounts of vitamin A ethers needed for sulfhydryl inactivation could be reduced materially with longer periods of incubation.

The *in vivo* depilatory action of the ethers shows an interesting parallelism with their other toxic effects. The methyl ether and palmitic ester cause depilation and anomalous ossification, while the phenyl ether is inert in both respects (18). It is conceivable that the phenyl ether cannot be broken down in the body to the as yet unknown active degradation product which is believed to be responsible for the action of vitamin A (15).

The inhibition of epidermal keratin formation by hydroxenin offers evidence in favor of the theory advanced by us that in large amounts vitamin A has a nonspecific drug action. Hydroxenin has no vitamin A activity and yet it has the same local effect as vitamin A has in large doses. The ineffectiveness of oxenin as an anti-keratinizing agent is probably due to its unstable nature. Hydroxenin has some of the desirable qualities which we were seeking: it is stable, cheaper and cosmetically more desirable than vitamin A. It is not advocated that this compound be used locally instead of vitamin A; our work merely indicates that the therapeutic effects of vitamin A may be achieved with other unsaturated compounds as well. Testing of such compounds is in progress.

On the basis of our present work, the following theory may be formulated concerning the mode of action of vitamin A in cutaneous metabolism: Vitamin A in excessive doses has a non-specific direct drug effect upon the epidermis; this effect is probably connected with interference with cutaneous sulfhydryl metabolism. It appears likely that the anti-keratinizing action is due to some unknown metabolite of vitamin A, since related compounds, like hydroxenin, have a similar action. Whether or not the physiologic effects of vitamin A in cutaneous metabolism are due to a similar mechanism, remains the object of future studies.

Our incidental finding that amyl acetate solutions of the vitamin A esters develop a strong color in the presence of concentrated hydrochloric acid, makes possible the development of a new colorimetric method for the quantitative

estimation of vitamin A. Preliminary experiments indicate that the color follows the Beer-law over a wide range. This new method has the advantage over the Carr-Price estimation that the developed color is stable. The sensitivity of the method is somewhat low, but can be increased. Work to develop this method is in progress and will be reported elsewhere.

SUMMARY

1. The mechanism of the "anti-keratinizing" (scale-preventing and depilatory) action of large doses of vitamin A was studied *in vitro* and *in vivo*.
2. It has been claimed that the local and systemic depilatory effects of vitamin A may be due to different mechanisms. In order to study the effect upon hair growth of orally administered unsaturated compounds, another unsaturated, local depilatory compound, oleic acid, was given to rabbits by mouth. When administered in large doses, oleic acid caused scaling and reversible hair loss on the ears of the animals. The baldness had the same features as the local depilation produced by oleic acid.
3. In large amounts, the methyl, phenyl ethers and palmitic ester of vitamin A inactivated sulfhydryl groups and the sulfhydryl enzyme succinic dehydrogenase. The methyl ether caused hair loss in mice after local application, while the phenyl ether was inactive.
4. Hydroxenin and oxenin, synthetic precursors in the manufacture of vitamin A, had the same *in vitro* effects as the ethers of vitamin A.
5. Diminution of scaling in patients with ichthyosis was observed after local application of hydroxenin ointment. Oxenin had no effect.
6. These findings support our previous supposition that in large amounts vitamin A has a direct, local, non-specific action on the epidermal cells. This effect is probably due to interference with the sulfhydryl metabolism of the epidermis. The active principle may be an as yet unidentified intermediary metabolic degradation product of vitamin A.

ACKNOWLEDGMENTS

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DISCUSSION

DR. STEPHEN ROTHMAN, *Chicago, Illinois*: In his remarkable series of experiments, Dr. Flesch has conclusively demonstrated that the biological effect of Vitamin A on the epidermis is due to a certain chemical configuration and that other compounds not having vitamin action but the same chemical grouping have the same effect. Thereby he has shown that this is not a vitamin effect.

I wonder if the picture he has shown us was actually a case of ichthyosis vulgaris because it showed involvement of the antecubital fossa while in ichthyosis vulgaris this fossa is never involved. Also I wonder if the therapeutic effects achieved with local application of vitamin A have any clinical significance. Can't as good results be obtained with any ointment base containing low concentrations of salicylic acid?

I also would like to ask whether it is not an over-simplification to attribute the complex and amazing metaplasia of keratinizing embryonic epithelium into a ciliated mucus-producing mucous membrane, as shown by S. Fell, to inhibition of sulfhydryl function. Is there any reason to assume that mucous membrane cells require less sulfhydryl-dependent enzymes than do the epidermal cells?

DR. FREDERICK REISS, *New York, N. Y.*: I have been not only impressed by the excellent contributions of Dr. Flesch, but added impetus was given to me by Dr. Sabella's investigations, (*Proc. Soc. Exp. Biol. & Med.* **76**: 499, 1951). The topical application of vitamin A to oophorectomized rats resulted in an average increase in epidermal thickness to about twice normal.

About two years ago, I investigated with Dr. Campbell (Department of Plastic Surgery, New York University Medical School) the effect of locally applied vitamin A to the senile skin. We have not used large doses as Dr. Flesch,

but only 3,000 vitamin A palmitate per ounce. In most patients, an appreciable improvement was seen in the dryness of the skin, scale formation, and follicular hyperkeratosis, which was not observed to such a degree in the control side using the base alone.

In relation to Dr. Rothman's question, I may be able to give an explanatory answer. The effect of vitamin A is clearly seen in obvious histologic changes. Our slides have been recently reviewed by Dr. Harman of the Department of Anatomy, New York University Medical School, who found in the vitamin A treated skin, noticeable changes in contrast to the controls. These changes consist mainly of the presence of many cells with pyknotic nuclei and an increase of keratohyalin granules. Pucinelli (*Giorn. ital. dermat.* 83: 840, 1948) applied vitamin A containing ointment in cases of lichen planus and psoriasis, and although no clinical improvement was recorded, histologic changes are similar to those observed in our investigations.

DR. MARION B. SULZBERGER, *New York, N. Y.*: In a carefully studied but still all too small series of cases Dr. Leah Fisher in our Department at the Skin and Cancer Unit has as yet been unable to show any regular substantial effect of locally applied vitamin A on the scaling of cases of ichthyosis. I don't know what the reason for the failures may be. I believe that Dr. Fisher's cases were all males and there may be a sex determined difference in response. In carefully controlled observations with contralateral comparison with blank control vehicles, no diminution of scaling, no clinical improvement could be observed in certain cases. In histologic studies, which are still unfinished and not yet ready for any definitive statement her careful (and tedious) planimetric measurements of the thickness of the stratum corneum and of the other layers of the epithelium evidenced no regular effects of the vitamin A inunctions when compared with control sites inunctioned with vehicles alone. Maybe Dr. Fisher is here and will want to say something about the present status of her planimetric and clinical studies.

Then I would like to call attention to some remarks that appeared in the introduction of the Yearbook of Dermatology and Syphilology for the years 1938 and 1941. These stated that the systemic administration of vitamin A was being effective in a large series of dermatoses characterized by abnormal keratinization, either follicular or non-follicular. The effect was not due to overcoming a dietary vitamin A deficiency—because the doses needed were far in excess of that which one would expect if this was a dietary vitamin effect, i.e. the doses necessary were far in excess of the daily dietary requirements. Moreover, the patients that received these benefits had no other evidence whatsoever of either a dysvitaminosis or a dietary vitamin deficiency.

DR. R. E. MAYER, *Summit, N. J.*: Dr. Flesch's investigations are of great theoretical interest because they seem to explain the role of certain lipids and especially unsaturated fatty acids in the skin. So far we have always looked at the skin lipids as serving for the lubrication of the hair and the skin. But it is quite possible that lipids, especially those rich in unsaturated fatty acids play an active role in skin metabolism. As with vitamin A, they may form redox systems as in

the case of many proteinic enzymes, and are actively engaged in the metabolism of energy-rich products. We were able to show in our own experiments that bacterial lipids, which have long been considered as simple protective agents play such an active role.

DR. SAMUEL M. PECK, *New York, N. Y.*: I have been stimulated for a number of years by Dr. Flesch's experiments. Since the initial experiments which we carried on in Darier's disease and ichthyosis our findings seemed at variance. Once was that while we believed that in a disease like Darier's we produced our vitamin A effect because here we felt was an abnormality in metabolism of vitamin A, we were unable to find either blood levels or other indications for such an assumption in ichthyosis. Yet we too noted that large doses of vitamin A, both locally and parenterally seemed to affect the ichthyotic skin favorably.

Insight into the effects of vitamin A in large doses in ichthyosis and other conditions with hyperkeratosis is now explained by Dr. Flesch's experiments. I believe, however, that diseases which have hyperkeratosis as a manifestation can be divided into two distinct types. (1) One is that we have a real dyskeratosis so that a receptor mechanism for vitamin A is abnormal. Such cases actually need relatively smaller doses to obtain effects than those in which we have ordinary hyperkeratosis with a normal vitamin A metabolism. (2) In ordinary follicular keratosis which is not due to a proven vitamin A deficiency or an abnormality in vitamin A metabolism even very large doses of vitamin A may cause no effects, or will give little, if any, changes. Skin diseases which are characterized by excessive parakeratosis fall somewhat in between these two groups. An example of this group is psoriasis. I have no reason to believe that an abnormal vitamin A metabolism plays a role in psoriasis. However, intramuscular injections of vitamin A in fairly large doses will cause a drop in blood levels in some psoriatics in contradistinction to the usual rise in normal patients. At the same time, the injections of vitamin A may favorably influence the course of psoriasis in some instances.

DR. THEODORE CORNBLEET, *Chicago, Ill.*: I presume Doctor Flesch refers to the nonspecific effects of vitamin A on the skin as action other than that of normal physiological keratinization. This latter power must be by indirect means, because we found no vitamin A as such on or in the normal skin. Doctor Flesch felt massive doses of oleic acid spilled over on the skin. Are there iodine number studies to corroborate his opinion? Doctor Peck pointed out that in Darier's and Devergie's diseases the blood vitamin A level may be normal. We found not only this may be true of the blood, but also the vitamin A stores in the liver are normal in both diseases.

DR. PETER FLESCH (*in closing*): I would like to thank those participating in the discussion for their kind remarks.

In answer to Dr. Rothman, the picture of alleged ichthyosis shown was indeed the antecubital fossa of a patient, diagnosed as ichthyosis simplex. We should have classified her as congenital ichthyosiform erythroderma. I am inclined to

believed that ichthyosis is used as a general term under which we discuss diseases which may look somewhat similar, but are probably different in their pathogenesis.

Fell's work is unfortunate in one respect, at least as far as dermatologists are concerned: she worked with chick embryos and not with mammalian skin. I don't think one can expect the same changes to occur in the epidermal cells of the mucous membranes of mammals as has been observed by her in the chick embryo. In the mouse, Dr. Fell found inhibition of hair formation *in vitro*, a study which is just briefly mentioned in her present work.

Inhibition of sulfhydryl groups may be limited to certain specific sites. For example, X-rays primarily inactivate the sulfhydryl groups of the cells of the hair follicles; vesicants affect mostly the sulfhydryl groups of the capillary endothelium. The solubility of the inhibitor may be a decisive factor in this respect.

I cannot recommend the routine use of local vitamin A ointments. First of all, this type of treatment is expensive. Then the results often are not better than one could obtain with other methods. I am not at all convinced, that, with the exception of some cases, the local treatment with vitamin A offers any advantage over more conservative methods.

In answer to Dr. Sulzberger's comment about failure of local vitamin A in ichthyosis, I wish to point out that in my original work I described six patients treated with local vitamin A in two of whom no beneficial effect was seen. As far as the mode of action of vitamin A is concerned, I could quote Dr. Sulzberger as supporting both the idea that vitamin A acts because of an alleged local hypovitaminosis ("dysvitaminosis") or because it has a pharmacological effect. To the best of my knowledge, the latest statement on this subject has been made in the Yearbook of 1949 (p. 13-14). In the same paragraph it is mentioned that acne may be due to a localized high requirement for vitamin A or vitamin A may have a purely pharmacological effect in this condition.

I am very grateful for Dr. Mayer's remarks and would like to call his attention to the fact that similar speculations about the possible enzymatic role of vitamin A have been raised in the past. It has been observed that when yeast cells were grown under high oxygen pressure, they were able to synthesize vitamin A, while normally they were not able to do so. (J. Exp. Cell Res., **1**: 494, 1950). This may indicate that under certain conditions yeast acquires an enzyme system which requires vitamin A for its functioning.

We did not carry out iodine number determinations of surface fats. Such work has been done in the past by Perutz et al. (Arch. Derm. u. Syph., **170**: 511, 1934) who injected cod liver oil into rabbits and observed decreased hair growth with an increase in the iodine number of the skin fats.

Concerning the physiological and pharmacological effects of vitamin A, I may have been too rigid in the past in order to drive a point home. A strict distinction between these two effects may be impossible. The two effects may overlap to a certain extent. After all, the physiological effect must take place through some chemical reaction and it is possible that the so-called drug effect is merely an accentuation of a normal physiological effect.

Effects of Dietary Fat on Mammary Carcinogenesis by 7,12-Dimethylbenz(α)anthracene in Rats¹

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SUMMARY

A semisynthetic high-corn oil diet enhanced the development of mammary cancer induced by 7, 12-dimethylbenz(α)anthracene (DMBA) in intact female Sprague-Dawley rats. This was in comparison to two other groups of similarly treated rats fed a high coconut oil and a low-fat semisynthetic diet, respectively. The average daily caloric intake was similar, and the average growth rate, based on body weight, was comparable in all three groups.

Fatty acid analyses demonstrated that, at the time of DMBA administration, the composition of mammary fat was different in the three different groups, reflecting their dietary fat intake.

The data from this study suggest that dietary effects upon DMBA mammary carcinogenesis were related to the nature as well as the amount of fat used.

INTRODUCTION

The fat content of diet has been found to influence carcinogenesis in a variety of circumstances. Tannenbaum in 1942 (15) obtained a higher incidence and shorter latent period of spontaneous mammary tumors in mice maintained on high-fat as compared to those on low-fat diet. A similar effect was observed also in mice, both with benzpyrene-induced skin cancer and to a lesser degree with spontaneous hepatoma (16). These effects were confirmed later on by Boutwell *et al.* (1) and by Silverstone and Tannenbaum (13, 14). Tannenbaum concluded that such effects were related to a specific action of dietary fat rather than to a general caloric influence (16, 17). This was based on the observation that the effects of changing the fat content of the diet were confined mainly to mammary and skin tumors, whereas caloric restriction *per se* inhibited other tumors as well (15). Moreover, when the same low caloric intake was maintained, augmentation of the dietary fat content tended to overcome the inhibitory influence upon mammary tumorigenesis (16).

A similar effect was also obtained in rats. Dunning *et al.* in 1949 (6) presented evidence that high-fat diet increased the yield and shortened the latent period of stilbestrol-induced mammary cancer in comparison with an equicaloric but low-fat diet. Tumors which appeared in the former group had a faster growth

rate. In 1951, Engel and Copeland (7) found that the incidence of mammary tumors induced by 2-acetylaminofluorene in female rats on high-fat was greater than in those on low-fat diet. This effect was specifically related to tumors of the breast and not to those induced in other organs by the same carcinogen. As far as spontaneous breast tumors are concerned, Davis *et al.* (5) reported, that in a group of rats allowed to live their usual life span, the incidence was increased from 57 to 80% when maintained on high-corn oil diet. As usual, tumors appeared late in life and 95% of them were of mammary origin. However, this diet did not increase the proportion of malignant tumors which formed only 12% of the total number of tumors in these rats.

The above-mentioned observations led Tannenbaum and Silverstone (17) to suggest that dietary fat could influence carcinogenesis by its effect on tissue lipid. It was postulated that the mechanism of action may be due to alteration of the rate of transfer of a carcinogen to its site of action and/or to an independent effect on the developing tumor cell. In 1959, Dao *et al.* (4) speculated that mammary adipose tissue functions as a storage depot for the carcinogenic hydrocarbon and that its proximity to the glandular epithelium facilitates further exposure of the latter to its carcinogenic action.

The relationship between dietary lipids and carcinogenesis in general was discussed by Haven and Bloor (9) and by Tannenbaum and Silverstone (17).

The purpose of this paper is to present the influence of dietary fat content upon the fatty acid composition of mammary tissue and its relationship to chemical induction of mammary cancer in rats.

MATERIALS AND METHODS

Animals. All were female, intact Sprague-Dawley rats of weaning age (21-23 days), and of an average weight of 48.8 gm \pm 2.85 (S.D.). At the beginning of each experiment, they were allocated to 3 different groups, each containing animals of comparable weight range.

Diets. From the day of weaning, the animals were maintained on 3 different semisynthetic diets (Table 1). The first group received a low-fat diet containing 0.5% corn oil as a vehicle for fat-soluble vitamins, the second and third groups, a high-fat diet containing 20% coconut oil and 20% corn oil, respectively. The caloric value of the low-fat diet was approximately 3.8 Calories (kilocalories)/gm, while that of each of the high-fat diets was 4.7 Calories/gm.

Diets and water were given *ad libitum* throughout the experiments. However, 2 days prior to carcinogen administration,

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TABLE 1
Composition of Semisynthetic Diets^a

Ingredients	Low fat (gm)	High coconut oil ^b (gm)	High corn oil ^c (gm)
Casein	18	23	23
Dextrose	72	46	46
Fat	0.5	20	20
Salt mixture ^d	4	5	5
Cellulose	5	5	5

^a Adequate amounts of fat-soluble vitamins (A, E, and K) and water-soluble vitamins (including choline and inositol) were added to each diet.

^b Coconut oil, Nopco Chemical Canada Ltd., Hamilton, Ontario, Canada.

^c Corn oil, St. Lawrence Starch Co. Ltd., Port Credit, Ontario, Canada.

^d Phillips-Hart salt mixture, Nutritional Biochemicals, Cleveland, Ohio.

all rats were switched to a standard laboratory ration³ and water for a total period of 3 days, at the end of which time they were returned to their respective semisynthetic diets. The purpose of this temporary change in diet was to minimize any possible effect of dietary fat on absorption of the carcinogen from the gut. The diets were weighed daily, and the approximate weights of ingested diet were recorded.

Carcinogen. At the age of 50–52 days, each animal was given a single dose of 10 mg of 7,12-dimethylbenz(α)anthracene (DMBA) in 0.5 ml of sesame oil by stomach tube.

Observation and Autopsy. Following DMBA administration, the rats were weighed and palpated for tumors twice a week. The observation period extended to 4 months, at the end of which time the animals were killed. At autopsy, the skin and subcutaneous tissue were reflected, and all tumors and suspicious subcutaneous lesions were resected and identified microscopically. Only those tumors which fulfilled the histologic criteria of adenocarcinoma were included in the results.

Fatty Acid Analyses of Mammary Tissue. These were performed in order to demonstrate that, at the time of tumor induction, a change had already taken place in the composition of the mammary tissue fat of rats in the 3 different diet groups. The animals used for analysis were submitted to diet schedules identical with the ones described above and were killed on the day corresponding to that of DMBA administration. The 4th pair of mammary glands was resected and saponified by refluxing with a mixture of 30% aqueous KOH and absolute ethanol, 1:3 (v/v). The hydrolysate was then acidified to a pH of 2.0 with concentrated HCl and the precipitated fatty acids were extracted with hexane and methylated by refluxing with 5% HCl in methanol (2). An aliquot of the fatty acid methyl esters was analyzed by gas-liquid chromatography (Barber-Colman Model 10). The column (6-feet × ¼-inch) was packed with 10% ECNSS-S (Applied Science Laboratories, State College, Pennsylvania) on Gas Chrom P (8) and operated at a temperature of 175°C. The main

³ The ration was Master Fox Cubes, Maple Leaf Mills Ltd., Toronto, Ontario, Canada.

peaks were identified by comparison with National Institute of Health Standard Mixtures. The fatty acid composition of each analyzed aliquot was calculated on the basis of weight percent of total fatty acids.

The fatty acid compositions of coconut oil and of corn oil used in the diets were determined for comparative purposes, using a method similar to the one described above.

RESULTS

Food Intake and Growth Rate of Rats. The average daily food intake per rat of the 3 groups is shown in Chart 1 (*lower graph*). It is obvious that the weight of low-fat diet ingested was higher than that of the 2 high-fat diets. However, when the average daily caloric intake per rat was calculated, it was found to be similar in the 3 groups as demonstrated in the upper graph of Chart 1. This was approximately 49.9, 49.2, and 47.0 Calories daily per rat on low-fat, high-coconut oil, and high-corn oil diet, respectively, between the 25th and the 74th days after weaning. The average amount of protein ingested daily during the same period ranged between 2.3 and 2.4 gm per rat on all diets.

The growth rate of these animals, based on body weight, was comparable in the 3 different diet groups (Chart 2). The intragastric administration of 10 mg of DMBA/0.5 ml of sesame oil at the age of 50 days, was followed in all groups by a temporary weight loss and growth retardation which lasted 3–4 days, at the end of which the animals resumed their steady growth.

Fatty Acids of Mammary Tissue. The results of gas-liquid chromatographic analysis of fatty acids are given in Table 2. The average weight of each was expressed as a percentage of the total fatty acids.

It was evident from these results that the fatty acid composition of the breast tissue was different in rats of different diet groups. That of rats fed a high-corn oil diet (mainly unsaturated) underwent an increase in unsaturated fatty acids, particularly in the linoleic acid fraction. The tissue of rats fed a high-coconut oil diet, had a decrease in unsaturated with an increase in saturated fatty acids. Lauric and myristic acids, both supplied by coconut oil of the diet, were especially elevated in this group. On the other hand, rats fed a low-fat diet, where the only external source of fat was 0.5% corn oil, exhibited a fatty acid composition intermediate between the other 2 groups with a relatively higher unsaturated fatty acid content, mainly due to an increase in the oleic acid fraction.

Effects of Diet on Mammary Carcinogenesis. Table 3 provides a detailed presentation of these results. A total of 22 rats were included initially in each of the 3 diet groups. However, the early loss of 1 animal from the low-fat and high-coconut oil groups reduced the effective total number to 21 rats in each.

Enhancement of mammary carcinogenesis in the high-corn oil diet group is detectable in most of the parameters studied. The overall tumor incidence, as indicated by the percentage of all tumor-bearing rats, was higher in the high-corn oil diet group than in the other 2 groups, but the difference was not statistically significant. However, when the incidence of palpable tumors, i.e. the percentage of palpable tumor-bearing rats in each group, was considered, it was found to be 57.1 and 66.7% in the low-fat and high-coconut oil groups respectively and 95.5% in the high-corn oil group, an increase over the 2 former groups which

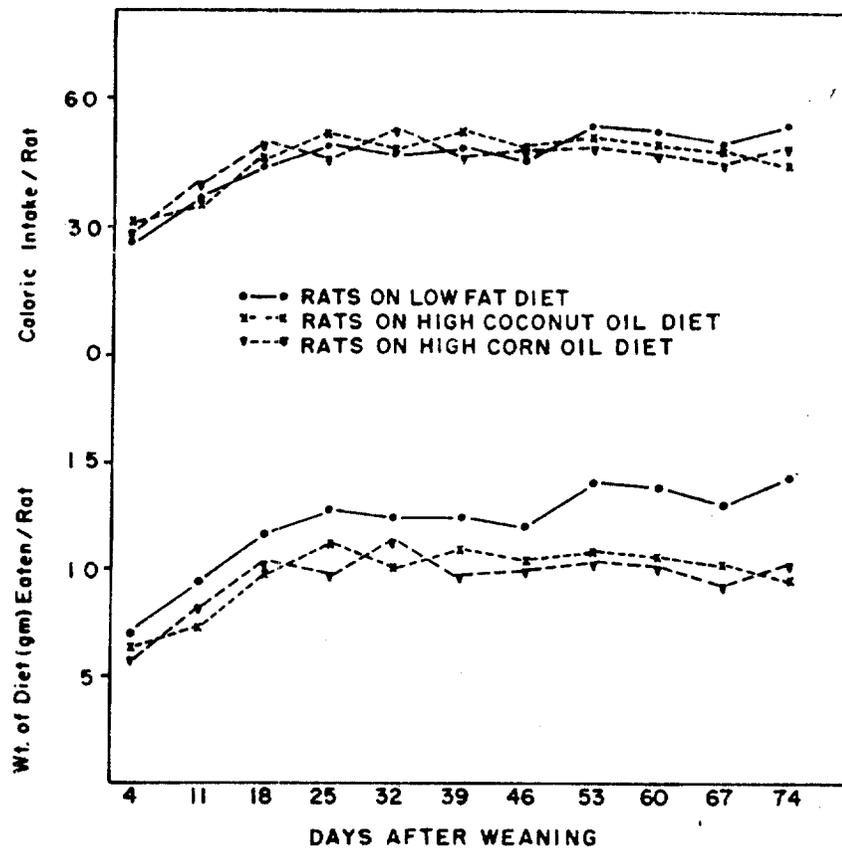


CHART 1. Lower graph, average daily food intake per rat of 3 semisynthetic diet groups, determined at weekly intervals. Upper graph, corresponding average daily caloric intake of same rats. Animals were given indicated diets from weaning. Thirteen animals in each group.

was statistically significant. It appeared, therefore, that tumor development was enhanced in the high-corn oil diet group so that the number of rats developing palpable tumors within the observation period was larger compared to the other 2 groups.

The average number of tumors induced per rat was greatest in the high-corn oil-fed group, which also had the highest yield of palpable tumors per rat, thus denoting once more a general tendency to an accelerated tumor growth in that group. These last 2 parameters were statistically significant at the 2% and 5% levels, respectively. When the comparison was confined to tumor-bearing rats, the average number of tumors per rat of the high-corn oil group was 4.8 as compared to 3.1 in each of the other 2 groups. Although not statistically significant, this increase nevertheless agrees with the general trend of enhanced mammary carcinogenesis in high-corn oil-fed rats. This was illustrated once more in the average latent period which was found to be shortest in this same group.

In all the above mentioned parameters, low-fat and high-coconut oil-fed rats showed a great similarity in the results obtained. When present, differences between these 2 groups were not statistically significant.

DISCUSSION

Since the purpose of the present study was to compare dietary effects, the use of semisynthetic diets was preferred to that of

commercial diets, whose composition depended on factors outside our control. Furthermore, it was shown that the 3 groups of animals under study had an equicaloric dietary intake and ingested approximately the same amount of protein in each case.

The dose of DMBA was reduced to 10 mg, half of the optimal tumor-inducing dose recommended by Huggins *et al.* (10), in view of the fact that DMBA is a compound of relatively high carcinogenic potency. Given in full dose, its effect would have masked any moderate influence of the dietary alterations under study [(17); E. B. Gammal, unpublished experiments].

Changes in the fatty acid composition of mammary tissue obtained in these experiments reflected the fat content of the 3 different diets on which the animals were maintained. Fatty acids that had the most affected concentrations were those not endogenously synthesized. Thus, linoleic acid underwent a marked elevation in the corn oil diet group while lauric and myristic acids showed relatively high levels in animals on coconut oil diet. On the other hand, palmitic, palmitoleic, stearic, and oleic acids were found to be present in the tissues of the three different groups. They are known to be endogenously synthesized. A relatively higher concentration of these acids (especially oleic) was detected in breast tissue of animals on low-fat diet, since their exogenous fat supply was minimal.

These findings are in agreement with those obtained in other studies concerning the effects of dietary fat on the fatty acid composition of animal tissue, as reviewed by Carroll (3). Thus,

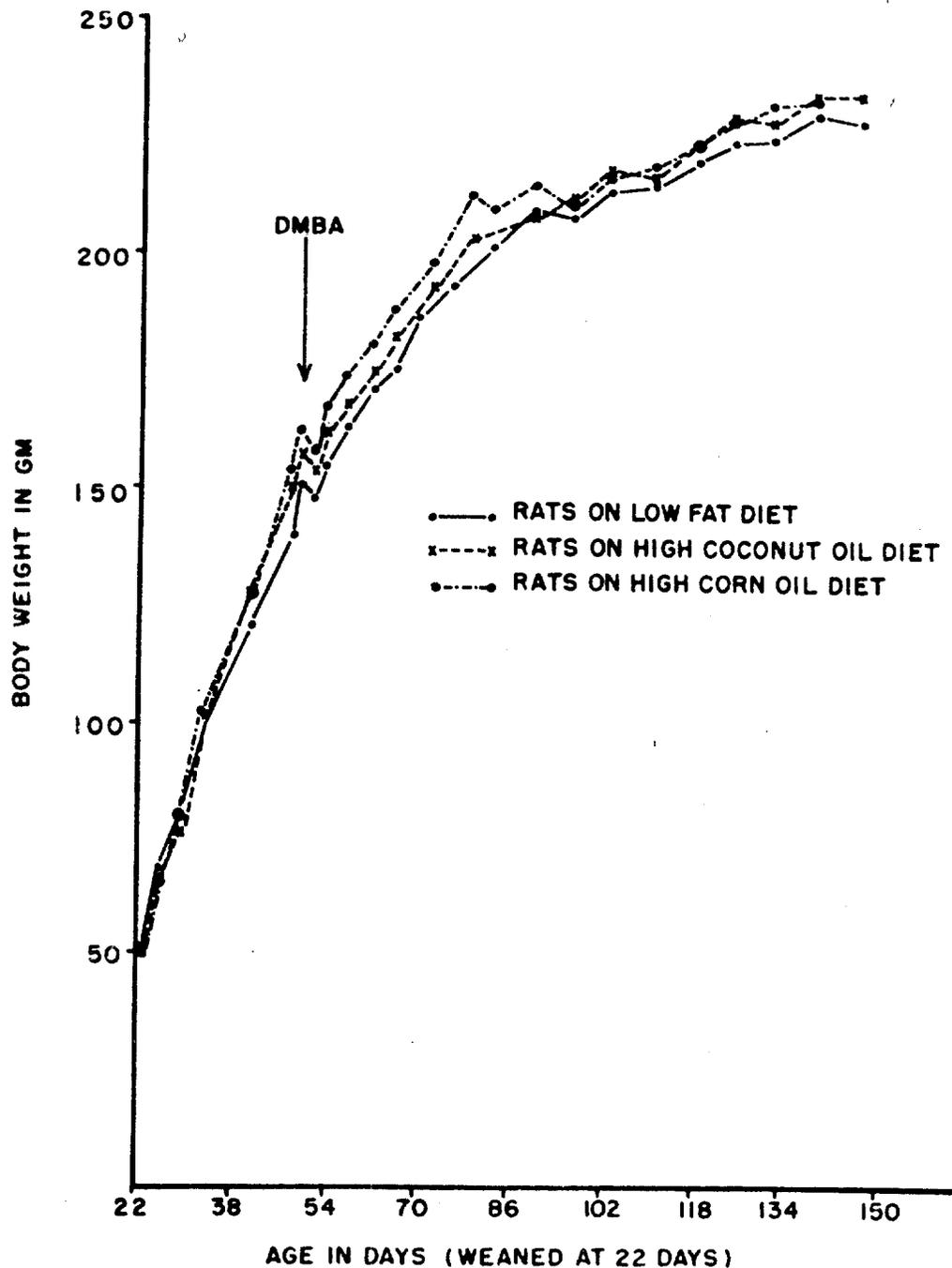


CHART 2. Growth rate of rats maintained on 3 different semisynthetic diets. Average of 13 animals in each group. *DMBA*, 7,12-dimethylbenz(α)anthracene.

important to the purpose of these experiments, it was found that the above-mentioned changes in the composition of mammary fat were present at the time of *DMBA* administration.

As regards *DMBA* mammary carcinogenesis, the results of the present experiments agreed with those of other investigators concerning the enhancing effect of high-fat as compared to low-fat diet on certain varieties of spontaneous and induced cancer in animals (6, 7, 11, 13, 15, 16). However, it was very significant to observe that such an enhancing effect was limited to high-corn oil diet, while the substitution of coconut oil for corn oil gave a

tumor yield which was generally as low as that obtained in the low-fat diet group. It was thus demonstrated that not only the amount but also the nature of dietary fat could have an effect upon *DMBA*-induced breast cancer in rats. This observation is reminiscent of the protective effect of hydrogenated coconut oil against the induction of rat hepatoma by *p*-dimethylanilinebenzene (12).

This finding also lends support to the suggestion that the effect of dietary lipids upon the formation of certain tumors is not merely a product of an increased caloric intake (13). Coconut

TABLE 2
Fatty Acid Composition of Mammary Tissue of Rats on Different Semisynthetic Diets

Fatty acid color	Shorthand designa- tion ^a	Percentage composition				
		Rat mammary tissue ^a			Commercial fat ^b	
		Low-fat diet	High- coconut oil diet	High- corn oil diet	Coconut oil	Corn oil
Lauric	12:0		8.3		41.1	
Myristic	14:0	1.3	11.9	1.0	30.0	
Palmitic	16:0	28.5	29.5	19.9	14.4	9.3
Palmitoleic	16:1	6.7	6.5	2.8		
Stearic	18:0	6.2	3.5	3.3	4.9	2.2
Oleic	18:1	52.0	35.1	33.0	6.5	26.9
Linoleic	18:2	4.6	4.7	39.5	3.1	61.7

^a Figures represent average values obtained using the fourth pair of mammary glands from 5 rats in each diet group.

^b Fatty acid composition of commercial fat is included for comparison.

^c First figure denotes the number of carbon atoms and second figure the number of double bonds in the fatty acid.

oil, presumably providing the animals with a comparably equal source of energy as that of an equicaloric intake of corn oil, did not exert the latter's enhancing influence upon the formation of DMBA-induced mammary tumors.

Since the growth rate of the animals and their caloric intake were comparable in all 3 groups, it would seem that the effects of altering either the quantity or the nature of dietary fat, as observed in these studies, were not related to nonspecific causes such as inanition or underfeeding, but rather to some specific influence of the fat itself.

One such influence, already demonstrated, was on the fatty acid composition of mammary tissue which was found to be different in the 3 different groups. Such a change in the nature of the fat, which constitutes the immediate environment of the mammary parenchyma, could either modify the latter's response to the carcinogenic action of DMBA and/or affect the availability of the carcinogen to the target cells.

In relation to the second possibility, it is hoped that a study of

the uptake and clearance of DMBA by breast tissue of rats, similar to those included in the present experiments, will be reported in the near future. Further discussion of the effect of dietary fat upon DMBA mammary carcinogenesis will be provided then, in the light of these results.

ACKNOWLEDGMENTS

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TABLE 3
Effects of Dietary Fat upon 7,12-Dimethylbenz(α)anthracene (DMBA) Mammary Carcinogenesis

Diet	No. of rats	Tumor incidence		No. of tumors ^a		No. of tumors ^b per tumor-bearing rat	Latent period ^c
		Total tumors	Palpable tumors	Total tumors	Palpable tumors		
1. Low fat	21	71.4%	57.1%	2.2 ± 0.46 ^d	1.5 ± 0.40 ^d	3.1 ± 0.47 ^d	76.2 ± 7.95 ^d
2. High coconut oil	21	76.2%	66.7%	2.4 ± 0.38	1.6 ± 0.32	3.1 ± 0.33	69.5 ± 6.63
3. High corn oil	22	95.5%	95.5%	4.6 ± 0.84	3.0 ± 0.52	4.8 ± 0.86	56.5 ± 3.68
<i>t</i> values for Diets 1 and 3		> 0.05 to <0.1	<0.01	<0.02	<0.05	> 0.1 to <0.2	<0.02
<i>t</i> values for Diets 2 and 3		> 0.1 to <0.2	<0.05	<0.02	<0.05	>0.05 to <0.1	>0.05 to <0.1

^a Tumor-bearing as well as nontumor-bearing animals were included.

^b Palpable as well as nonpalpable tumors were included.

^c Days from DMBA feeding until appearance of first palpable tumor.

^d Mean ± S.E.

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ACTION OF ANTIBIOTICS

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The change in features of antibiotics' pharmacological action and the mechanisms of their influence on the organism led not long ago to the discovery of the so-called adaptogenic action of these preparations. The nature of such action is that during the extended administration of different antibiotics, adaptation of the organs and tissues to their action is observed, which is accompanied by an increase in the organism's resistance to intense (stress) pathogenic factors of various natures: hypoxia, infection, radiation sickness, etc. (1-3).

Judging from data in the literature, nonspecific adaptogenic action usually arises distinctly during the administration of tetracycline. As is known, this antibiotic possesses the ability to accumulate in mitochondria and to release oxidation phosphorylation (4-6). The question arises of the degree to which the ability of tetracycline to increase cellular immunity may be connected with its releasing action. Is this action realized, as described mainly in in vitro experiments, during the adaptogenic effect of the antibiotic on the whole organism? Is it a releasing action on the respiratory chain under certain conditions for the existence of the adaptation process or could it be absent with the use of antibiotics of another type? In the present article an attempt is made to answer these questions.

PROCEDURE

The experiments were conducted on white mice weighing 20-25 g. The animals received tetracycline or levomycetin daily (intramuscular, 2000-2500 units). The control mice received a physiological solution. On the day after the final administration of the antibiotic, a direct determination was made of tetracycline content in the tissues. After 24 hours the animals were subjected to mitochondrion extraction or were used to determine resistance. A radicimetic material, oxidized oleic acid, was used as the resolving toxic agent (intraperitoneally administered, up to 0.04 ml). This dose caused the death of the animals after 3 days. The ratio between the number of living and dead mice in the experiment to the control group determined the degree of the animals' resistance. The change in oxidation phosphorylation was observed in liver mitochondria using the polarographic method (7). The reaction mixture consisted of 20 millimoles of K_2PC_4 , 10 millimoles $MgCl_2$, 10 millimoles succinate, 15 millimoles HCl , and 0.2 millimoles of mannitol solution, 0.07 moles of sucrose. The final volume of 1 ml ADP in 200 or 300 millimoles was administered in the experiment as a mitochondrion supplement. Respiration rate of the specimens was compared in the presence and absence of the acceptor. Respiration rate was determined on segments of the polarographic curves,

corresponding to 30-60 sec. intervals, and it is expressed in microatoms of oxygen in seconds per l. To evaluate the stimulating action of ADP on the operation of the respiratory chain, a method was utilized which had been used earlier in our laboratory (8), the comparison of the effects of greater and less concentrations of acceptor in different combinations with substrates (functional load).

In order to decrease the value of seasonal factors and possible fluctuation in the experimental procedure, the measurement of mitochondrium respiration was carried out every day of the experiment on the control mice and on the animals receiving antibiotics.

RESULTS OF THE STUDY

The experiments indicated that toward the end of the first week of antibiotic administration, the sensitivity of the experimental animals to oleic acid does not change. After two weeks of antibiotic use, a distinct increase in resistance is observed. In one series of experiments after the administration of oleic acid, only 25% of the animals died which had received tetracycline, and 75% of the controls. In another series, 50 and 100 %, respectively, died. (The variation in the absolute value of these quantities is due to the fact that in different series, oleic acid of differing degree of acidity was used, therefore of unequal toxicity.) The death of those animals which received tetracycline occurred in both series over a more prolonged period than the death of the control animals.

Thus our experiments, in conformity with data from the literature (2), showed that for the adaptogenic action of tetracycline to occur, a definite period (up to 2 weeks) was needed for the antibiotic effect on the organism.

The study of the effect of ADP on mitochondria respiration showed that in this period an essential change takes place in the operation of the respiratory chain which deepens in relation to the increase in period of antibiotic effect on the organism. These results are given in Fig. 1. The diagrams in the right-hand vertical series (see Fig. 1) represent the values calculated on the respiration curves for breathing control, which was measured as the ratio of the respiration rate of the specimen with the acceptor to the respiration rate of the specimen without acceptor. It is evident that even a brief administration of tetracycline to the animal strongly influenced the operation of the mitochondrium respiratory chain. The stimulating action of ADP for both concentrations tested decreased from 2.4 and 4.2 to 1.8 and 2.3, respectively. A comparison of the graphs for various days shows that the decrease in breathing control deepens with an increased period of tetracycline action on the animals. It is expressed especially sharply after a 16-day administration of the antibiotic to the point of increased resistance.

It is necessary to turn our attention to the fact that, during this decrease in breathing control, it is more significant not only in absolute value but also in the ratio of the stimulating action of greater and lesser ADP concentration. In the mitochondria of the control animals and after the first days of tetracycline administration, a noticeable increase is observed in stimulating action of ADP during the transition from a lower concentration to a higher one. After 1 week of the antibiotic's effect, such an increase is barely observable. Finally, in the mitochondria of animals which received tetracycline for 16 days, a reverse ratio

was often noted. Only in the first few days of 3×10^{-4} mole ADP action was a higher rate of respiration observed than with the action of 2×10^{-4} mole ADP. However after 10-20 sec. of incubation, this originally higher rate decreases, and, in the remaining period of measurements, it became lower than the respiration rate in the presence of 2×10^{-4} mole ADP.

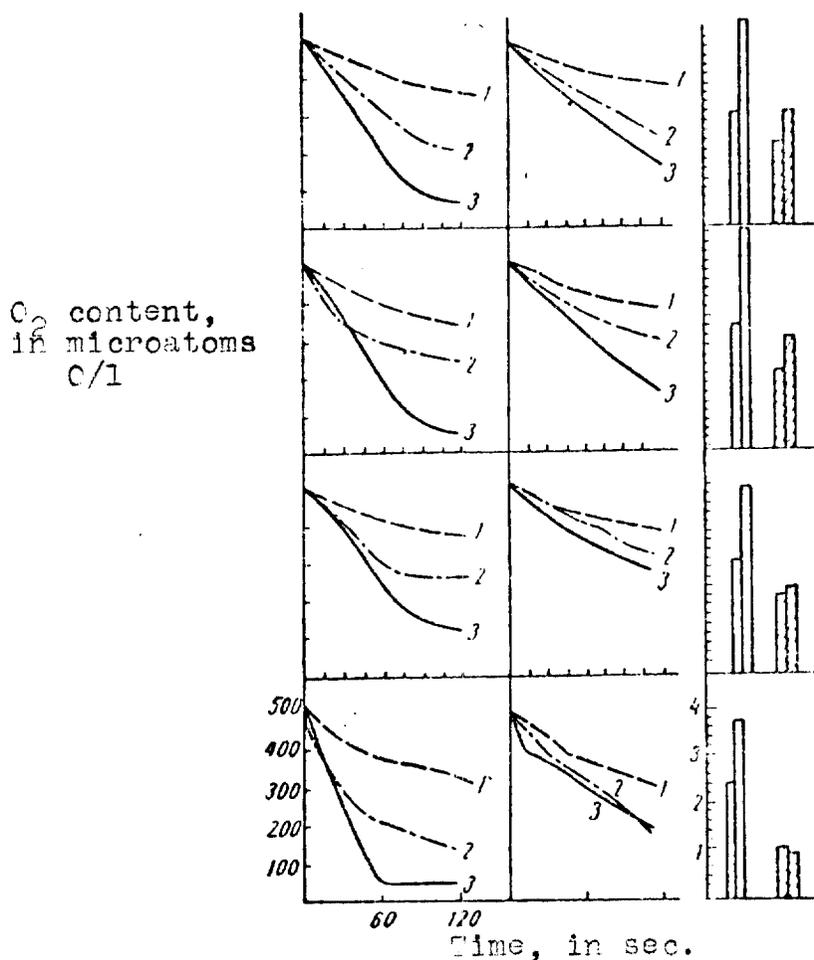


Fig. 1. Change in reaction to ADP of the respiratory chain in mouse liver mitochondria during tetracycline administration.

Horizontal series (from top to bottom): data for 1, 3, 7, and 16-day tetracycline administration; left vertical series: data on oxygen requirements for these respective periods for the control animals; middle series: data on oxygen requirements for animals which received tetracycline.

Diagrams of the right-hand vertical series: values of ADP respiration stimulation based on a comparison of samples without additional acceptor for controls and test animals; 1) respiration without ADP; 2) respiration with addition of 2×10^{-4} mole ADP; 3) respiration with addition of 3×10^{-4} mole ADP.

Light-colored columns: stimulation of mitochondrion respiration of control animals for greater and lesser ADP concentrations (left to right); dark-colored columns: stimulation of mitochondrion respiration in test animals for lesser and greater ADP concentrations (from left to right).

Thus, after 16 days of tetracycline administration, a value of 1.1 was observed in the test animals for the lower ADP concentration and 1 for the higher concentration, instead of the values of 2.5 and 3.8 observed for respiration stimulation in the control animals, for 2×10^{-4} and 3×10^{-4} mole ADP respectively. So the whole range of change in respiration reaction to ADP in proportion to tetracycline administration is especially great for the higher of the test ADP concentrations, since the 3×10^{-4} mole ADP stimulates greater respiration in the mitochondria of the control animals than does the 2×10^{-4} mole. After 16 days of tetracycline administration, the reverse occurs.

Fig. 1 also shows that, in addition to a great change in respiration which ADP stimulates, respiration without the acceptor essentially does not change during tetracycline administration. As special determinations showed, the amount of albumin in the mitochondrium preparations does not change either. Both these circumstances lay the basis for assuming that the reorganization of mitochondrium function (observed as an effect of tetracycline) is associated with a change in the regulatory action of ADP, and not with a decrease in the amount of respiratory agents. The phenomena described are not caused by the presence of tetracycline in the mitochondrium, since their absence in the liver was established as dependent on the length of the course of injection, by direct determination as early as the next day after the end of dosage administration. (As was noted above, the experiments lasted 2 days after the last antibiotic administration.) The effect of tetracycline on the state of resistance and on the respiratory chain is retained in the animals for some time after the cessation of antibiotic injection.

After the 16-day tetracycline administration, such an after-effect was delayed 6-8 days. During this period a decrease in respiratory control of ADP was observed in many of the animals which received tetracycline, a change especially notable during the transition from lower to higher ADP concentrations. (The examples of several cases for the controls and the animals "resting" from tetracycline are presented in Fig. 2.)

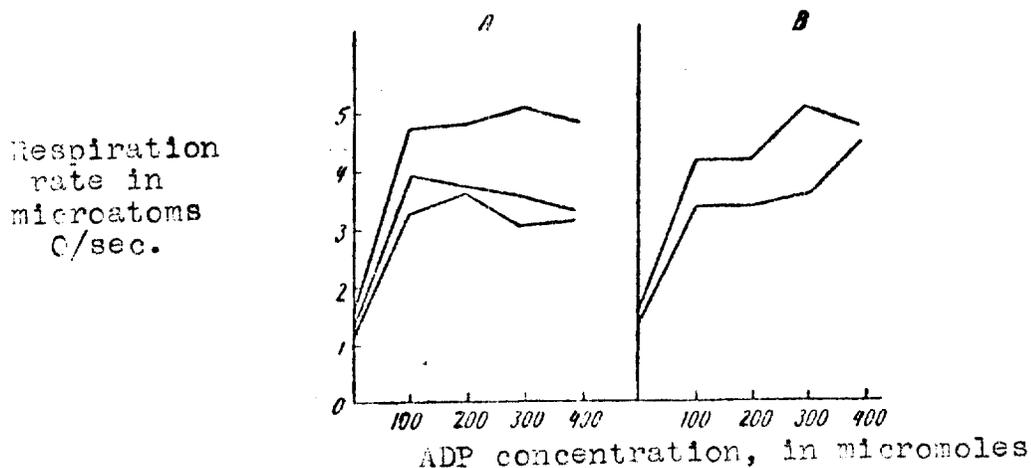


Fig. 2. Examples to mitochondrium respiration in animals which have "rested" 6 days after the 16-day tetracycline administration. A) test animals; B) control

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conditions of our experiments, a 2-week administration of antibiotics succeeded in yielding an increase in resistance to oleic acid only during the action of tetracycline and only for that time when it elicited a significant decrease in the stimulating action of ADP on mitochondrium respiration. The administration of levogysetin did not lead in our experiments to an increase in resistance, and it evoked no change in respiratory chain reactions. Thus, the impression is gained that the strong decrease in the stimulating effect of ADP on respiration (observed within the period of increased resistance and which appears especially sharp during its increase in concentration) is a necessary condition for an increase in immunity to the toxic effect of oleic acid. However such a change alone in reaction to ADP is evidence, obviously, only of the limits of the operating potentials of the respiratory chain. It would seem that such a sort of mechanism must cause a more rapid decrease and not an increase in immunity of the organism to stress stimuli. However it can be submitted that the matter proceeds in the following way. Considering the well-known releasing action of oleic acid (10,11), it is obviously necessary to evaluate the increase in the amount of oxygen, observed during its administration, which is required for a unit of ADP (the decrease in ADP/C), as an unfavorable phenomenon in the functional ratio. This high-intensity respiration must have a rapidly attained, low P/C , guaranteeing a small yield of energy products and at the same time seriously depleted tissues. Evidently, in such a situation the limited, low-effect, and depleting respiration proves to be unfavorable for the cell. The experiments conducted showed that this was so and that it provides for the preliminary prolonged effect of tetracycline on the animals.

It seems to us that the changes in the respiratory change lie at the basis of the mechanism of decreasing the stimulating action of ADP under the influence of tetracycline, close to those which are responsible for the occurrence of the phenomenon of respiration inhibition by ADP, in agreement with the data of Chance and Hagihara (12). This inhibition, conducted in their experiments, as in ours, with the use of succinic acid, was elicited by an increase in oxidized NAD content in a pyridine-nucleotide base along with succinate. Oxidation of NAD in this base at a somewhat more critical level led to the actual acceleration of oxalacetic acid formation, an inhibitor of succindehydrogenase. As a result of this, it led to the relaxation of oxygen requirements.

It is very probable that during prolonged administration of tetracycline, the oxidized NAD content in the respiratory chain gradually increases by virtue of its releasing action, which leads to an increase in formation of oxalacetic acid in the animals' mitochondria. These changes in the respiratory chain of the mitochondria of animals which received tetracycline are also manifested in a decrease in response to ADP addition, which occurs more sharply the greater the concentration of ADP used. In this presentation a comparison was made of the kinetics of oxygen requirements of mouse liver mitochondrium, in our experiments, in the presence of 3×10^{-4} mole ADP after 10 days of tetracycline action, with experiments done by Chance and Hagihara during ADP addition to "aged" mitochondria of a dove's heart. In both

cases, a short-term increase in respiration is observed which alternates with a sharp rate slowdown. In the period of mutual acceleration, increased formation of oxalacetic acid may take place, and more so with greater ADP addition or the greater the original degree of oxidized NAD in the mitochondrial preparation studied.

Such a mechanism of respiration inhibition may lie at the basis of the adaptogenic action of tetracycline in relation to other stimuli. An excessive increase in external gas volume and tissue respiration, stimulated by a sharp increase in adrenal function, is a complex part of the organism's reaction to strong effects of different kinds (13, 14). Thus, the unfavorable action of tetracycline on the mitochondrion which limits the operation of the respiratory chain potential can by itself serve as a useful adaptive reaction during stress effects.

CONCLUSIONS

1. Tetracycline administration to mice for 16 days increases the immunity of animals to a fatal dose of oleic acid.
2. The prolonged administration of tetracycline is associated with a progressive decrease in the stimulating action of ADP on liver mitochondrion respiration. This decrease is especially significant for higher ADP concentrations and reaches its highest level after 16 days of antibiotic administration with adaptation.
3. Administration of a minute dose of oleate to mice increases the respiration of isolated mitochondria, lowering the ADP/C coefficient. This effect of oleate is weakened by preliminary administration of tetracycline to the animals.

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TESTING CARCINOGENICITY OF EDIBLE OILS.

(Part) I.

PEANUT OIL.

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REPEATED confirmation of mutagenic effect of vegetable oils and edible fats, especially pea-nut and mustard oils on plant tissue (Sikka and Swaminathan, 1956; Swaminathan and Natrajan, 1956, 1959), warranted careful investigations on carcinogenicity of these substances. The oils are reported to induce chromosome breakage and viable mutations in plant cells. Successive somatic mutations have been suggested as the basis for the evolution of the cancer cell and may be the final common pathway for the activity of many carcinogens (Burnet, 1957; Fischer, 1958; Colter and Ellem, 1960). In the light of this hypothesis, pea-nut oil of proven mutagenic effect is tested for probable carcinogenic effect on animal tissue.

MATERIAL AND METHODS.

The first sample of pure pea-nut oil was received from the Indian Agricultural Research Institute, New Delhi. Further studies were continued on refined pea-nut oil (Sepoy Brand) from Swastik Oil Co. Ltd., Bombay.

Hybrid mice of XVII \times C57 (black) strain were used as test animals. This strain of mouse has proved to be a suitable strain for screening a weak carcinogen on its skin (Ranadive *et al.*, 1963). The treatment was started between the age of 10 and 12 weeks and animals were observed till death or were sacrificed when they appeared weak and emaciated. A total number of 97 mice was used in these studies. A small group of 24 Wistar strain rats was also used for testing early effect of the oil.

EXPERIMENTS.

The oil was administered by four different routes :

1. Cutaneous application. 2. Subcutaneous injection. 3. Intraperitoneal injection. 4. Feeding by stomach tubing. Experimental groups were arranged as follows :—

To test pea-nut oil sample received from the Indian Agricultural Research Institute, New Delhi :

Group I.—Single subcutaneous injection of 0.5 ml. of the oil to nine mice.

Group II.—Seventeen mice were fed 0.05 ml. of oil daily (except on Sundays) by stomach tubing for 3 months.

To test Sepoy Brand (from Swastik Oil Co. Ltd., Bombay) refined pea-nut oil :

Group III.—Daily cutaneous applications of oil were given by No. 5 camel hair brush on the interscapular region of 12 mice.

Group IV.—In 12 mice croton oil was used as a co-carcinogen in association with pea-nut oil. The oil was administered on the skin four days per week and 3 per cent croton oil in liquid paraffin once a week. Both the groups III and IV received the treatment till death.

Group V.—Four subcutaneous injections of 0.5 ml. of oil were given to 10 mice at monthly interval. Total dose was 2 ml. per animal.

Group VI.—Five intraperitoneal injections of 0.2 ml. of oil were given to 12 mice at monthly interval. Total dose per animal was 1 ml.

The animals from groups V and VI were kept under observation till death.

Group VII.—Twelve mice were fed daily 0.05 ml. of oil throughout their life except on Sundays.

Group VIII.—An untreated control group of 13 mice was observed till death.

A short-term experiment was carried out on 24 Wistar rats, to study the early effect of the oil feeding on the liver.

Group IX.—Twelve Wistar strain rats were fed by stomach tubing daily 0.1 ml. of oil for six months. These were observed for one more month after the treatment was over and were sacrificed. Body weights and liver weights were taken.

Group X.—Twelve untreated rats were killed as control at the same age as that in group IX. Body-weights and liver weights were recorded.

The mice from groups I to VIII were sacrificed when they appeared weak and emaciated. Site of treatment, all visceral organs, lungs and gonads were observed for gross abnormalities. Tissues at the site of treatment and remote, suspected to have undergone changes, were fixed for histopathological study.

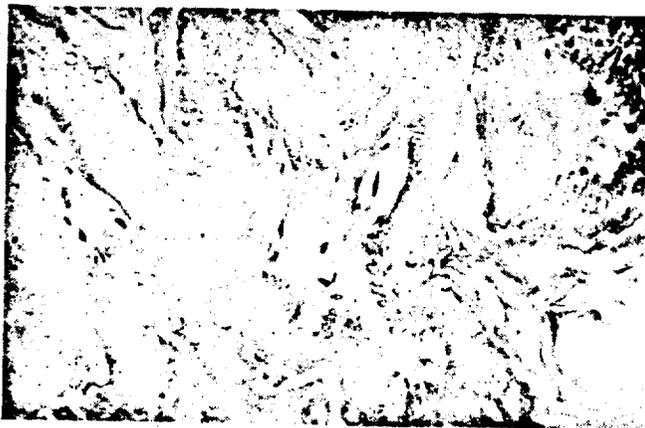
EXPERIMENTAL FINDINGS.

In the cutaneous application groups (III and IV), slight thickening of epidermis and black pigmentation was observed with co-carcinogen. In the group treated with just oil even thickening of skin was not observed.

In the subcutaneously injected groups (I and V), deposition of oil or its metabolites was seen in the subcutis even 22 months after termination of treatment. In histological preparations these areas showed only little inflammatory reaction.

In intraperitoneally injected animals (group VI), only one of the twelve animals had abnormal liver.

In the animals fed with oil (groups II and VII), no malignant lesion was found in the digestive tract but two of the twelve animals from continuously fed group had well developed papillomatous growth in the inner wall of the cardiac end of the stomach (Plate I.IX). In untreated control group, also, there was one papilloma.



Photomicrograph of papilloma on the inner wall of stomach at cardiac end. Animal was fed with 0.05 ml. of peanut oil (Sepoy Brand) daily for 23 months (group VII). $\times 230$

TABLE I.
Summary data of pea-nut oil testing for carcinogenicity.

Sample of oil	Route of administration.	Number of animals	ABNORMALITIES :					Malignancy.
			Skin.	Liver (necrotic fatty changes).	Spleen (atrophy).	Stomach (papilloma at the cardiac end).	Lymph node (enlarged).	
I.A.R.I., New Delhi.	Control	13	..	1	6	1	6	..
	Single subcutaneous injection	9	5 oil deposition.	2+1 hepatoma.*	2	..
	Oral administration for 3 months	17	..	4	5	..	3	..
Sepoy Brand (refined) Bombay.	Cutaneous application	12	..	1	3	..*	1	..
	Cutaneous application with co-carcinogen	12	6 Hyperplastic.*
	Multiple subcutaneous injections	10	8 oil deposition.	..	2	..*	1	..
	Multiple Intra-peritoneal injections	12	..	1	3	..*	2	..
	Continuous feeding	12	1 papilloma.	1	3	2	1	..

* Stomach wall not examined.

Other abnormalities found in this series were necrotic liver, atrophic spleen, and enlarged lymphnodes. These were also observed to a certain extent in untreated control group (*vide* Table).

In the short term experiment (groups IX and X), the liver weight per 100 g. body-weight in oil fed rats was 3.3 g. and that in the untreated control rats was 3.4 g. There was no difference found in the liver weights of treated and control rats. There was no gross abnormality observed to warrant histological study.

DISCUSSION.

Reported mutagenic effect of pea-nut oil on plant tissues (Swaminathan and Natrajan, *loc. cit.*) are worth careful attention. These reports prompted testing of oils for carcinogenic effects on animal tissues. Various routes of administration were, therefore, tried in large groups of mice and careful long-term studies were undertaken. Results confirm that the I.A.R.I. sample of pea-nut oil and refined pea-nut oil (Sepoy Brand) have no carcinogenic effect on animal tissues. There was no evidence of even weak carcinogenic activity in the group treated with the co-carcinogen croton oil. Nothing of carcinogenic significance was observed either at the site of administration or on the remote organs. The minor abnormalities observed in the tissues cannot be positively attributed to the oil treatment. The oil has been used before by others as a solvent for other test substances screened for carcinogenicity. The solvent control groups of animals treated with pea-nut oil have been reported to exhibit no carcinogenic effect (Hartwell, 1951). The present data confirm pea-nut oil to have no carcinogenic effect on animal tissues.

SUMMARY.

1. Refined pea-nut oil has been tested for its carcinogenic activity on XVII × C57 (Black) hybrid mice. Four different routes of administration, namely (i) cutaneous application, (ii) subcutaneous injection, (iii) intraperitoneal injection, and (iv) oral feeding have been used for testing the oil. No evidence of malignancy was observed at the site of the treatment or in the remote organs of experimental animals.
2. Early effect of oil feeding on liver was also investigated in a small group of Wistar strain rats. No gross lesions of the liver were discovered.

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COMPOSITION OF THE FATTY ACIDS CONTAINED IN LIPIDES
EXTRACTED FROM OIL SEEDS AND FRUITS

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Premise

The consumption in Italy of oils and fats of vegetable origin, for alimentary and industrial use (excluding olive oil, which is not the object of our investigation), has increased notably in recent years, passing from 1,414,000 liters in 1952 to 2,988,600 in 1962, as seen from the data published by the Central Statistics Institute and reported by the "Cereal Market" (1).

The Italian oil industry has contributed to this increased consumption in a significant manner, increasing production from 669,600 q.l. in 1952 to 2,254,500 in 1962 (2).

The vegetable oils most used are the following: soybean (595,134 q.l), colza and rapeseed (442,110 q.l), peanut (357,250 q.l), linseed (225,072 q.l), grapenut (190,152 q.l), maize seed (160,056 q.l), sunflower seed (129,538 q.l), sesame seed (101,595 q.l), ricinus (44,508 q.l), and to a lesser degree the oils extracted from tomato seeds, cotton seeds, rice buds, hemp and mustard seeds (53,647 q.l total).

Vegetable fats (normally used for the preparation of margarine and edible fats) are those of the coco palm, palm, illipe, palmisto and shea (661,244 q.l total).

The data cited refer to the oils and fats consumed in Italy during 1962 and obtained from oil seeds and fruits of both national and foreign origin. (1).

With the intention of contributing to our knowledge of the composition of the fatty acids contained in vegetable oils and fats, we have reported in tables 1 and 2 data obtained by means of gas chromatographic analyses, this technique being considered the most appropriate (James, 3; Lipsky, 4; Liberti, 5; Pallotta, 6; Wolff, 7) for determining the exact nature and percentage of the fatty acids present in the lipides.

The research was conducted following the suggestions reported in relation to olive oil, which has by far been the most studied of the vegetable oils. In fact, various authors (Vitagliano et al., 8; Garoglio and Boddi Giannardi, 9; Paolini and Pascucci, 10; Kaderavek, 11; Rotini et al. 12; Grieco, 13; Montefredine and Laporta, 14; Synodinos et al., 15) have studied, using vapor-phase chromatography, the nature and have determined the composition of Italian and foreign olive oil, while to our

knowledge, the authors who, using the same method of examination, have extended the study to other vegetable oils and fats are relatively few.

Toward the end of searching for new sources of fatty acids essential to human nutrition, Cescon and Giovetti determined the composition of the oil extracted from seeds of onion (16) and tomato (17); Mikolajczak et al. (18) occupied themselves with the oils of crucifers, and Galoppini and Lotti with the oil extracted from almonds (19) and pumpkin seeds (20).

Experimental Portion

The analysis was conducted on 80 oils and fats obtained from 36 species of oil seeds and fruits obtained from places of production as indicated in tables 1 and 2.

The fatty material was extracted from oil seeds and fruits with petroleum ether in accordance with the method reported in the Italian Standards for the control of Fats and Derivatives (N.G.D.) (21). The acidity of the oil or the fat (manifested by oleic acid) was determined by the method described in the above-mentioned Standards. The results are reported in columns 5 and 6 of tables 1 and 2.

The methyl esters for the gas chromatographic analyses were obtained by trans-esterification with methyl alcohol and sodium methylate when we were dealing with oils and fats with acidity less than 3%, while, when the acidity exceeded this value, we proceeded to extract the fatty acids and to esterify them with methyl alcohol and sulfuric acid (22).

The gas chromatographic analyses of the methyl esters were done with a gas chromatograph Fractovap model B/f of the firm of Carlo Erba in Milan, and the experimental conditions were the following: temperature of the thermostatic chamber 213°C, of the evaporator, 305°C, transport gas, pure helium with a flow of 100 ml/75" stationary phase composed of polyethylene-glycol succinate 20% on two different bases (celite C 22, 60-80 mesh and chromosorb W, 60-80 mesh). The columns were of unoxidizable steel, two meters long, with an internal diameter of 6 mm and the volume of methyl esters injected was equal to 2-4 microliters.

The percentage of the individual fatty acids was calculated with the method of triangulation. Due to the lack of a sufficient variety of pure methyl esters, we were not able to proceed to a special phase of the gas chromatograph we were using; nonetheless, it was possible, on more than one occasion, to compare our data with those obtained on identical samples by researchers whom we have mentioned (Capella, Laporta, Pallotta, Vitagliano), finding an optimum agreement.

Results and Discussions

The percentual content of oil or fat extracted from oil seeds and fruits and the acidity are reported in columns 5 and 6 of tables 1 and 2.

In table 1 are listed the fatty acids found in the oils extracted from oil seeds and fruits, and in table 2 the fatty acids present in the solid fats of vegetable origin.

Reviewing the data reported in table 1 and relating either to samples of seeds of various species or seeds of one species but of different origin, the following considerations are possible.

Peanut oil

The content of linoleic acid varies according to the origin of the seed from 17.0% to 35.7%; that of oleic acid from 40.4% to 63.4% of the total fatty acids, while their sum is found between 75.4% and 80.4% of the total fatty acids.

Linolenic acid was absent in all the samples analyzed with the exception of those obtained from Rhodesia, where the acid is present in traces.

Finally, the presence of a relatively large quantity of beenic acid (2.3%-3.6%) and lignoceric acid (0.7%-1.5%) is worthy of note.

Oils of seeds of crucifers (colza, rape, mustard)

All the oils of this species have erucic acid (minimum 39.0% - maximum 51.0%) as their principal component; an exception is the oil extracted from the seeds of Canadian rape, for which the major component is oleic acid (31.0%), followed in order by erucic acid (24.9%).

Particular attention is merited by the presence, in the crucifer oils, of tetracosenoic acid (C_{24} with a double bond), which was between 0.6% and 2.2% (table 1, column 24), and the high content in eicosenoic acid (C_{20} with a double bond), found between 7.6% and 11% (table 1, column 18).

Sunflower seed oil

The content in oil extracted from sunflower seeds depends upon the origin of the seed. Sunflower seeds cultivated in Bulgaria have an oil content constantly superior to that of the same seeds cultivated in Venetia*.

On the other hand, the nature and percentage of the individual fatty acids did not reflect different climatic and atmospheric conditions. The major component was linoleic acid, amounting to between 51.9% and 58.2% of the total fatty acids.

The two samples of seeds received from Egypt have an oil content of 27.0%-29.1% and a composition of fatty acids different from the former. In fact, in these, the chief component was oleic acid (49.5%-52.0%), while linoleic acid is equal to 37.0%-40.5% of the total fatty acids.

* The results of the analyses performed in preceding years on samples of Bulgarian seeds have median values of 46.0% equal to those reported in table 1. The Venetian samples have median values of 35.0%, and only in some cases of 43.0%. A seed of indigenous variety cultivated in the same zone yielded an oil content of 24.7% (reported in table 1).

Maize seed oil

Linoleic acid is the component with the highest representation of the fatty acids, and varies from 42.4% in white maize seeds to 52.5% in that of the yellow variety.

Linseed oil

At the end of the industrial utilization of linseed oil the notable variation of the linolenic acid content is still present (minimum 45.2% - maximum 58.3%). This variation is expressed commonly by the iodine number, which is between 170 and 195. According to some authors (27), these differences can depend on various varieties of seeds, the sowing time, the fertilization of the earth.

Sesame seed oil

Oleic and linoleic acid are the components chiefly represented in the oil extracted from sesame seeds. According to the origin of the seed, the percentual content varies for the first acid in relation to the second. In the oils extracted from seeds from Egypt, Mexico and Nigeria, linoleic acid is equal respectively to 44.0%, 44.6%, 47.4%, while in those from the Sudan, it is equal to 40.0% of the total fatty acids. Oleic acid, on the other hand, moves from 39.7% (Egypt) to 39.5% (Mexico) to 36.5% (Nigeria), to 42.0% (Sudan).

Soybean oil

Linoleic acid figures as the chief component from among the fatty acids (minimum 48.2% - maximum 54.9%). The presence of a slight quantity of linolenic acid (5.7%-10.0%) should be underlined.

Grapenut oil

The percentual composition of the fatty acids of the oil extracted from grapenut seeds differs notably from that of the oils hitherto examined with regard to the large value of linoleic acid, which is between 69.3% and 74.3% of the total fatty acids.

Oils extracted from various oil seeds and fruits

In considering the other vegetable oils we examined, we note that oleic acid was the major component of the oil extracted from seeds of the pistachio and cashew (60.8%), bitter almonds (70.6% and sweet almonds (72.0%), walnuts (83.5%), rice chaff (40.6%), and tea seeds (54.3%), while linoleic acid was the major component of the oil extracted from hemp seeds (59.4%), safflower seeds (75.4%), onion seeds (62.3%), cotton seeds (51.1%), hard corr kernels (53.6%) and soft corn kernels (58.3%), kenaph (49.0%), neuk (72.6%), nut kernels (61.5%), seeds of chili (75.0%) and tomato (55.4%), rice buds (42.0%), and finally from pumpkin seeds (42.3%). The oil extracted from ricinus seeds differs from all

the oils hitherto examined with respect to the ricinoleic acid content, which was equal to 83.3% of the total fatty acids.

From an examination of the results shown in table 1, particular attention is merited by lauric and myristic acid, which are notoriously slight in vegetable oils. In fact, lauric acid is present in some oils only in undosable traces, and myristic acid is also present only in traces or in a quantity equal to 0.1% of the total fatty acids. An exception are the oils extracted from kenaph seeds and chili beans, rice buds and punpkin seeds, in which myristic acid was equal to 0.2% and the oils obtained from rice chaff and cotton seeds, in which it was equal respectively to 0.3% and 0.9% of the total fatty acids.

There now remain to be examined the data relating to the solid vegetable fats enumerated in table 2; some of these, presenting particular analogies, are grouped as follows:

Fats of babassu, murumuru, coco and palmisto

A common characteristic is a high level of lauric acid, which is for all four the major component of the fatty acids; even taking into consideration their composition, it is always possible to distinguish them from each other. The fat of babassu, in fact, differs from the other three fats in that it has a lesser content of lauric acid, 44.5% as opposed to circa 50.0%; the peculiarity of coco is that it possesses a percentage of oleic acid that is nearly half that of the other three fats; in the palmisto are found a percentage of caprilic and capric acid that hovers around 4.0%, while the content of the same fatty acids in the babassu, coco and murumuru amounts to 6%-7%; this last fat, finally, is distinguished by a greater myristic acid content.

Fats of illipe and shea or karite

Stearic acid is their major component, and is about 48.0% in the fat of illipe and 45.5% in that of shea; these fats differ above all in a varying content of palmitic acid, which was on the average equal to 16.7% in the fat of illipe and only 3.7% in that of shea. Among vegetable fats, this last deserves particular mention for being the one with the highest level of heptadecanoic acid, (1.1%).

Mowrah fat

The principal component of the fatty acids was oleic acid, equal to 34.8%-35.3% of the total fatty acids.

Beyond this, this fat differs from the other solid fats of vegetable origin in its high linoleic acid content (14.5%).

Palm fat

Palmitic acid is the principal component (43.9-44.6%), followed in order by oleic acid (37.7%-38.0%) and linoleic acid (9.8%-10.7%).

Conclusions

Having had the opportunity of analyzing oils and fats extracted from oil seeds and fruits of certain origins, by means of gas chromatography, we have considered it opportune to publish the results obtained, hoping thus to be able to contribute, with this modest work, to a better knowledge of the composition of numerous oils and fats, and to be able facilitate the means of controlling the purity of some samples, or of establishing, if only approximately, the percentage of the individual components of the mixture of oils or fats.

The percentages of the single fatty acids found in the samples we examined are in agreement with those reported by other researchers (16, 17, 18, 19 and 20), who used the same technique of analysis, while sometimes relevant differences are noted in relation to the compositions reported by Hilditch (28) obtained, however, by means of different analytical methods.

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Table 3 (cont.)

Nome Comune	Famiglia e denominazione botanica	Lipidi totali % s.s.	Acidi % s.g.	ACIDI GRASSI %																							Insaturi saturi	oleico	linoleico	
				C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:0}	C _{20:1}	C _{20:2}	C _{20:3}	C _{22:0}	C _{22:1}	C _{22:2}	C _{22:3}	C _{24:0}	C _{24:1}	C _{24:2}	C _{24:3}	C _{26:0}	C _{26:1}						
Cipolla	Liliaceae Allium cepa	24,9	4,8	—	—	—	tr.	—	—	8,6	0,2	—	—	2,2	29,6	59,4	tr.	tr.	0,2	—	—	—	—	—	—	—	—	8,3	0,5	
		24,1	5,0	—	—	0,2	—	—	—	7,6	0,5	—	—	tr.	3,1	29,6	58,7	tr.	0,3	tr.	0,2	—	—	—	—	—	—	8,2	0,5	
Olivio semi (frutto)	Oleaceae Olea europaea	45,6	1,8	—	—	—	tr.	—	—	9,6	0,3	0,1	tr.	2,8	68,0	17,0	0,6	0,6	0,6	—	—	—	—	—	—	—	—	6,4	4,0	
		n.d.	n.d.	—	—	—	tr.	—	—	9,9	0,6	—	tr.	tr.	2,6	79,0	6,2	0,7	0,4	0,6	—	—	—	—	—	—	—	6,7	12,7	
Sedano	Ombrellifereae Apium graveolens	22,1	0,4	—	—	tr.	tr.	—	—	4,9	1,6	tr.	tr.	1,0	68,5	20,4	0,7	—	1,1	0,5	1,3	—	—	—	—	—	—	15,8	3,3	
		—	—	—	—	tr.	tr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Sesamo Egipt. Sudan. Messico	Pedaliaceae Sesamum indicum	61,5	0,7	—	—	tr.	—	—	—	9,4	0,2	tr.	—	6,0	40,6	42,4	0,4	0,8	0,1	—	—	—	tr.	—	—	—	—	5,2	1,0	
		53,2	0,8	—	—	tr.	—	—	—	—	9,8	0,3	tr.	—	6,2	41,0	41,0	0,5	0,7	0,2	—	—	—	tr.	—	—	—	—	5,0	1,0
		54,4	0,8	—	—	tr.	—	—	—	—	9,8	0,4	tr.	—	5,5	37,8	44,3	0,8	0,9	0,3	—	—	—	tr.	—	—	—	—	5,2	0,9
Pino (sesti)	Pinaceae Pinus pinaster	54,8	0,7	—	—	tr.	—	—	—	7,3	0,3	tr.	—	3,1	38,8	46,8	0,6	0,4	0,8	tr.	0,4	1,5	—	—	—	—	—	8,2	0,8	
		54,1	n.d.	—	—	0,1	tr.	—	—	—	6,6	0,3	tr.	—	3,7	40,8	44,2	0,6	0,4	0,8	tr.	0,4	1,7	—	—	—	—	8,2	0,9	
		55,0	n.d.	—	—	0,1	tr.	—	—	—	6,3	0,2	tr.	—	3,8	39,6	45,0	0,7	0,5	0,9	tr.	0,5	2,1	tr.	—	—	—	—	8,5	0,9
		55,8	n.d.	—	—	tr.	tr.	—	—	—	7,1	0,2	tr.	tr.	3,5	38,0	47,6	0,6	0,5	0,5	tr.	0,4	1,4	—	—	—	—	8,0	0,8	
Albicocca	Rosaceae Prunus armeniaca	43,2	0,5	—	—	tr.	—	—	—	6,3	0,8	tr.	0,1	1,7	60,6	30,1	0,3	tr.	tr.	—	—	—	—	—	—	—	—	11,5	2,0	
		—	—	—	—	tr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Mandarino	Rutaceae Citrus aurantium	57,6	0,2	—	tr.	0,1	0,1	tr.	tr.	6,4	0,6	0,2	0,1	2,4	69,4	18,4	0,8	0,4	0,6	—	—	—	—	—	—	—	—	9,4	3,8	
		55,5	0,3	—	tr.	—	—	—	—	—	6,1	0,4	tr.	tr.	1,8	72,8	18,8	tr.	tr.	tr.	—	—	—	—	—	—	—	—	11,6	3,9
Pesca	Rosaceae Prunus persica	31,1	1,1	—	—	tr.	—	—	—	5,9	1,0	tr.	tr.	1,2	63,8	28,1	tr.	tr.	tr.	—	—	—	—	—	—	—	—	13,1	2,3	
		38,9	0,3	—	—	tr.	tr.	tr.	tr.	tr.	6,1	0,4	tr.	0,1	1,8	69,0	22,2	0,1	0,1	0,1	—	—	—	—	—	—	—	—	11,5	3,1
Mela	Rosaceae Malus domestica	25,5	2,0	—	—	tr.	—	—	—	7,0	0,2	tr.	tr.	1,8	52,7	55,2	0,8	0,5	0,4	—	—	—	0,3	—	—	—	—	8,4	0,6	
		24,7	1,2	—	—	tr.	—	—	—	—	6,5	0,4	tr.	tr.	1,6	30,9	52,0	6,0	0,4	tr.	—	—	tr.	—	—	—	—	—	n.d.	0,6
Rosa canina sunt. int. sunt.	Rosaceae Rosa canina	n.d.	n.d.	—	—	0,3	0,1	tr.	0,2	4,8	0,3	tr.	tr.	3,6	17,7	53,6	19,4	—	tr.	—	—	—	—	—	—	—	—	10,1	0,3	
		6,8	1,7	tr.	tr.	0,3	0,1	tr.	0,2	4,9	0,4	tr.	tr.	3,7	17,6	53,2	19,6	—	tr.	—	—	—	—	—	—	—	—	9,9	0,3	
Caffè (torr.)	Rubiaceae Coffea arabica	15,5	2,8	—	—	tr.	—	—	—	35,6	tr.	0,2	—	9,3	9,2	41,1	4,3	0,2	—	—	—	—	—	—	—	—	—	n.d.	0,2	
		n.d.	n.d.	—	—	tr.	tr.	—	—	—	36,7	tr.	tr.	—	7,3	8,1	43,6	3,6	0,1	—	—	—	—	—	—	—	—	—	n.d.	0,2
Arancio	Rutaceae Citrus aurantium	n.d.	n.d.	—	—	0,1	—	tr.	tr.	26,7	1,3	0,4	0,2	5,0	23,9	38,5	4,4	tr.	tr.	—	—	—	—	—	—	—	—	2,2	0,6	
		n.d.	n.d.	—	—	tr.	tr.	tr.	tr.	tr.	22,2	0,8	tr.	0,2	3,8	30,7	32,9	9,1	tr.	0,2	—	—	—	—	—	—	—	—	2,8	0,9
Limone	Rutaceae Citrus limon	n.d.	n.d.	—	—	tr.	tr.	tr.	tr.	22,2	0,7	tr.	tr.	5,7	28,2	38,9	3,7	tr.	0,2	—	—	—	—	—	—	—	—	2,6	0,7	
		n.d.	n.d.	—	—	tr.	tr.	tr.	tr.	tr.	22,2	0,7	tr.	tr.	5,7	28,2	38,9	3,7	tr.	0,2	—	—	—	—	—	—	—	—	2,6	0,7
Mandarino	Rutaceae Citrus nobilis	n.d.	n.d.	—	—	0,1	—	tr.	tr.	26,7	1,3	0,4	0,2	5,0	23,9	38,5	4,4	tr.	tr.	—	—	—	—	—	—	—	—	2,2	0,6	
		n.d.	n.d.	—	—	tr.	tr.	tr.	tr.	tr.	22,2	0,8	tr.	0,2	3,8	30,7	32,9	9,1	tr.	0,2	—	—	—	—	—	—	—	2,8	0,9	
		n.d.	n.d.	—	—	tr.	tr.	tr.	tr.	tr.	22,2	0,7	tr.	tr.	5,7	28,2	38,9	3,7	tr.	0,2	—	—	—	—	—	—	—	2,6	0,7	
		n.d.	n.d.	—	—	0,1	—	tr.	tr.	tr.	26,7	1,3	0,4	0,2	5,0	23,9	38,5	4,4	tr.	tr.	—	—	—	—	—	—	—	—	2,2	0,6
		n.d.	n.d.	—	—	tr.	tr.	tr.	tr.	tr.	22,2	0,8	tr.	0,2	3,8	30,7	32,9	9,1	tr.	0,2	—	—	—	—	—	—	—	—	2,8	0,9
Melanzana	Solanaceae Solanum melongena	19,6	6,0	—	tr.	0,1	tr.	—	—	8,8	0,2	tr.	—	3,4	18,1	67,7	1,4	tr.	0,2	—	—	—	tr.	—	—	—	—	7,1	0,3	
		24,3	0,7	—	—	0,1	tr.	tr.	tr.	tr.	11,2	0,5	0,4	0,1	2,7	10,8	71,9	0,8	tr.	1,1	—	—	—	—	—	—	—	5,7	0,2	
		27,3	1,8	—	—	tr.	0,1	tr.	tr.	tr.	12,4	0,4	tr.	tr.	3,0	11,1	72,1	0,2	0,2	tr.	—	—	—	—	—	—	—	—	5,3	0,2
		25,4	0,7	—	—	tr.	0,1	tr.	tr.	tr.	12,5	0,4	tr.	tr.	3,2	11,6	71,3	tr.	0,3	tr.	—	—	—	—	—	—	—	—	5,1	0,2
		n.d.	n.d.	—	—	0,1	tr.	tr.	tr.	tr.	11,3	0,5	tr.	tr.	3,1	11,0	73,6	0,2	0,2	0,1	—	—	—	—	—	—	—	—	5,8	0,2
Pomodoro semi (S) buccola	Solanaceae Solanum lycopersicum	n.d.	n.d.	—	—	0,3	—	tr.	tr.	16,2	0,8	tr.	0,4	5,8	21,0	52,0	2,8	0,6	tr.	—	—	—	tr.	—	—	—	—	3,4	0,4	
		n.d.	n.d.	—	—	0,1	—	tr.	tr.	tr.	14,8	0,9	tr.	0,3	4,7	19,9	54,3	2,9	2,0	tr.	—	—	—	tr.	—	—	—	—	3,6	0,4

(segue)

Table 1. Composition of the oils extracted from oil seeds and fruits

Key:

1. Oil seeds and fruits
 - a. pistachio or cashew
 - b. peanut
 - c. hemp
 - d. safflower
 - e. onion
 - f. colza
 - g. cotton
 - h. sunflower
 - i. (1) hard corn (seed)
(2) soft corn (seed)
 - j. maize (bitter kernel)
(1) white maize (mill seed)
(2) yellow maize (mill seed)
 - k. kenaph
 - l. linseed
 - m. (1) bitter almonds
(2) sweet almonds
 - n. neuk
 - o. walnut
 - p. nut (kernel)
 - q. chili
 - r. tomato
 - s. rapeseed
 - t. ricinus
 - u. rice (bud and chaff)
 - v. mustard
(1) white
(2) black
 - x. sesame
 - y. soybean
 - z. tea
 - aa. grapenuts
 - bb. pumpkin
2. Botanical name
3. Family
4. Origin
 - a. Mozambique
 - b. South Africa
 - c. Italy
 - d. United States
 - e. China
 - f. Erythrea
 - g. France
 - h. Italy (Lombardy)
 - i. Italy (Venetia)
 - j. Sweden
 - k. Egypt
 - l. Ethiopia
 - m. Mexico

Key cont'd:

n. Brazil

5. Oil content (petroleum ether) %
6. Acidity of the oil (in oleic acid) %
7. Fatty acids %
 - a. Lauric
 - b. Myristic
 - c. Palmitic
 - d. Palmitoleic
 - e. Heptadecanoic
 - f. Heptadecenoic
 - g. Stearic
 - h. Oleic
 - i. Linoleic
 - j. Linolenic
 - k. Arachic
 - l. Eicosenoic
 - m. Eicosadienoic
 - n. Beenic
 - o. Erucic
 - p. Docosadienoic
 - q. Lignoceric
 - r. Tetracosenoic

tracce = traces non determinato = undetermined

Table 2 - Composition of fats extracted from oil seeds and fruits

Key:

1. oil seeds and fruits
 - a. babassu
 - b. coco palm
 - c. illipe
 - d. mowrah
 - e. murumuru
 - f. palm *traces of pentadecanoic acid
 - g. palmisto
 - h. shea or karite
2. botanical name
3. family
4. origin
 - a. Brazil
 - b. Philippines
 - c. West Africa
5. Fat content (petroleum ether) %
6. Acidity of the fat (in oleic acid) %
7. Fatty acids %
 - a. Caproic
 - b. Caprilic
 - c. Capric
 - d. Lauric
 - e. Myristic
 - f. Palmitic
 - g. Palmitoleic

Table 2 key -- cont'd:

- h. Heptadecanoic
- i. Stearic
- j. Oleic
- k. Linoleic
- l. Linolenic
- m. Arachic
- n. Eicosenoic

UNSATURATED FATTY ACIDS IN THE DIETARY DESTRUCTION
OF *N,N*-DIMETHYLAMINOAZOBENZENE (BUTTER YELLOW)
AND IN THE PRODUCTION OF ANEMIA IN RATS

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Hepatic injury and the occurrence of malignant hepatoma after ingestion of butter yellow were first seen in rats (1) fed a diet of which 95 per cent was rice and 5 per cent olive oil. To this mixture the butter yellow was added in the proportion of 0.06 gm. per 100 gm. This ration was supplemented daily with a small amount of carrots. In contrast, practically no malignant changes have been seen (2), at least up to 150 days of the experimental period, in the livers of rats fed a diet (called diet C) that contained butter yellow in the same proportion as the original rice diet (0.06 per cent) but that had a different composition, *i.e.*, casein 6 per cent, lard 23, cane sugar 15, cornstarch 50, salt mixture 4, and cod liver oil 2 and was supplemented daily with thiamine, riboflavin, pyridoxine, and pantothenic acid. The lesions in the livers of rats fed this diet were limited to necrosis, cirrhosis, and proliferation of the bile ducts, mostly typical in character, indeed only rarely atypical. Even these pathological changes were prevented to a large extent by the administration of a combination of cystine and choline (2).

Explanation of the difference in the effect of the rice-oil mixture and diet C on the production of malignant hepatoma in rats has been sought by feeding rats a modification of diet C. Crisco or melted butter fat was substituted for lard in one group of experiments and rice (brown unpolished or white) for cornstarch and sugar in another group of experiments. The effects of these substitutions proved in both sets of experiments to be procarcinogenic, especially in those in which brown rice was used. Furthermore, the malignant changes that followed ingestion of the rations containing butter fat or brown rice were not prevented, or were prevented only to a limited degree, by the administration of a combination of cystine and choline.¹

The problem that arose, therefore, was to learn what principle was operating

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¹ Detailed report of the pathological findings will be given later in cooperation with Dr. Harry Goldblatt of the Institute of Pathology, Western Reserve University.

in the change of the non-carcinogenic diet C into a carcinogenic ration following the incorporation in it of crisco or butter fat in place of lard in one set of experiments and of rice (white and especially brown) instead of cornstarch and sugar in another. The answer has been provided by results of still other experiments in which the source of fat was, first, a crude preparation of linoleic acid² and, later, pure specimens of fatty acids and their esters, prepared in the laboratory of one of us.

EXPERIMENTAL

The experimental results, representative examples of which are given in Tables I and II, can be summarized as follows:—

1. Diet C with crude linoleic acid replacing lard (referred to as diet "By" in Tables I and II) proved to be toxic for rats weighing over 130 gm. at the beginning of the experiment. Sixty-seven rats were fed this diet in amounts *ad libitum*, and the results were consistent. The animals lost weight rapidly in spite of satisfactory intake of food. They exhibited marked, progressive anemia, secondary in type, which was accompanied, as a rule, by leucopenia (Table I). They became infested with pediculi, often before the development of anemia when the animals were still very active and not nearly moribund.

The toxic effect on rats of the ration containing linoleic acid was even more pronounced when butter yellow was omitted from diet "By" (referred to as diet "Li" in Table I). In this group comprising 23 rats all the animals died before the 80th experimental day, with an average survival time of 55 days. Four rats showed leucopenia but no anemia before death.

The toxic effect of the rations containing linoleic acid with ("By") or without ("Li") butter yellow could, as a rule, be neutralized preventively and therapeutically by the daily addition of 0.5 to 1 gm. of yeast. Twenty-one rats were fed diet "By" and 11 rats were fed diet "Li." The observation period for these groups was extended to 150 days. In 3 of the 11 rats fed ration "Li" (without butter yellow) the anemia was not prevented by addition of 1 gm. of yeast daily, and the animals died before the end of the experimental period.

In tests involving 49 rats, a diet containing brown rice, linoleic acid, and butter yellow was found to be non-toxic. On the other hand, linoleic acid was almost completely oxidized and destroyed by keeping diet "By," before use, for 3 to 4 weeks at laboratory temperature, and the diet then exerted marked toxic effect on all 23 rats in this group. Diarrhea was a prominent feature, in addition to loss in weight, anemia, leucopenia, and pediculosis.

Postmortem examination revealed that rats fed the modification of diet C with linoleic acid instead of lard and with or without butter yellow were generally free from pathological changes in the liver, even from severe fat infiltration. Animals receiving diet C with linoleic acid and butter yellow never showed malignant hepatoma. The life span of these rats could be prolonged by daily

² "Linoleic acid refined light" from the Glyco Products Co., Inc., Brooklyn, N. Y.

TABLE I
Morphological Blood Changes in Rats Fed Rations Containing Crude Linoleic Acid

Rat No. and date when experiment was started	Diet*	Blood examination									
		Date	Hb	Red blood cells $\times 10^6$	White blood cells (corrected) $\times 10^4$	Reticulocytes	Nucleated red cells	Polymorphonuclears	Small lymphocytes	Large lymphocytes	Eosinophils
			per cent			per cent	per cent	per cent	per cent	per cent	per cent
7596—12/2	"By" + cystine + choline	2/18	72	5.7	9.4	0.3	1	20	76	4	..
		3/13	46	2.25	7.8	22.5	6	22	78
		4/3	36	2.6	4.5	5.6	20	12	88
		4/28	15	1.05	5.4	17.0	32	14	84	2	..
8063— 3/3	"By"	3/25	93	6.1	26.9	3.2	..	16	80	4	..
		4/17	70	6.35	27.0	0.9	6	12	88
		5/8	59	4.45	9.3	0.4	20	24	76
		5/19	16	1.1	6.8	—†	40	20	80
8132— 3/19	"By" + yeast	4/7	83	7.2	19.2	9.6	2	20	80
		4/30	88	7.35	12.5	2.6	2	36	64
		5/21	84	5.5	18.5	4.4	4	28	68	..	4
6995—11/17	"By" + cystine and beginning 1/29 + yeast	1/29	29	3.0	2.3	—†	2	34	58	8	..
		2/16	63	6.4	3.4	2.1	52	48	52
		3/31	78	5.7	14.8	7.6	..	28	72
		5/11	79	6.65	11.2	7.2	..	34	66
8007— 2/21	"Li"	2/23	94	8.1	15.2	0.3	..	8	88	4	..
		3/17	90	6.6	18.2	0.9	..	10	88	..	2
		4/21	45	4.55	4.5	1.2	2	20	76	4	..
		4/28	13	1.1	0.24	16.0	85	85	15
8011— 2/21	"Li" + yeast	3/20	92	8.0	12.8	9.6	..	24	76
		4/13	107	8.25	7.8	2.4	..	20	80
		5/5	103	8.4	19.0	0.2	..	14	86

* Diet "By:" casein 6 per cent, cornstarch 50, sucrose 22, cod liver oil 2, salt mixture 4, linoleic acid 16, and butter yellow 0.06. Diet "Li:" same as "By" without butter yellow. Both diet "By" and diet "Li" were supplemented daily with 20 μ g. of thiamine, 25 μ g. of riboflavin, 20 μ g. of pyridoxine, and 100 μ g. of pantothenic acid. Both rations were freshly prepared twice a week.

† Not examined.

supplements of cystine (50 mg.) or of cystine (50 mg.) plus choline (20 mg.). Only one of the 19 rats that did not receive supplements of cystine or choline survived for 100 days. The average survival time in this group was 61 days.

Again, only one rat in a group of 15 rats that received 20 mg. of choline daily survived for 100 days. The average survival time in this group was 56 days. In contrast, only 6 out of 20 rats receiving daily supplement of cystine (50 mg.) and 4 of 13 rats receiving daily supplement of cystine (50 mg.) plus choline (20 mg.) died before the 100th experimental day. Twelve rats in the first of these two latter groups and 6 in the second were killed at the end of 150 days of the experimental period. The supplements of cystine and of cystine plus choline, however, had no beneficial effect or had only limited beneficial effect on the production of anemia, leucopenia, and pediculosis.

2. The color of the modified diet C containing crude linoleic acid instead of lard, with butter yellow, underwent rapid change even at room temperature and almost completely disappeared after 4 days. In distinct contrast, a mixture containing rice (white or brown), linoleic acid, and butter yellow did not lose its color after 7 days and that with brown rice even after 20 days. Thus, rice appeared to stabilize butter yellow in the presence of linoleic acid.

Quantitative data are given in Table II concerning the iodine number and color of the chloroform extracts of different mixtures which contained, in varying combinations, butter yellow, sand, sucrose, cornstarch, rice starch, white or brown rice, casein, salt mixture, and, as a source of fat apart from crude linoleic acid, pure linoleic acid, arachidonic acid, oleic acid, palmitic acid, methyl palmitate, and methyl oleate.

For the determination of iodine numbers Hanus' (3) method has been used. The concentration of butter yellow in the chloroform extracts was measured by the Klett-Summerson photoelectric colorimeter, using the blue filter No. 42.

DISCUSSION

From the results obtained it is apparent that linoleic acid and, to a less degree, arachidonic acid and even less distinctly oleic acid are connected with the destruction of butter yellow *in vitro*, with a parallel drop in the iodine number of linoleic acid and arachidonic acid. It can be assumed that these unsaturated fatty acids, perhaps through formation of peroxides, decompose butter yellow and at the same time give rise to formation of toxic by-products which in turn produce in rats severe progressive anemia, leucopenia, and loss in weight, together with pediculosis, but leave the liver more or less unharmed. As has been mentioned, these toxic compounds are formed also in the absence of butter yellow.

The decolorization of butter yellow in the presence of linoleic acid is a surface phenomenon; it does not take place in a homogeneous oily mixture. It is accelerated by high temperature (+35°C.) and is retarded by low temperature (-20°C.). It is not observed in the absence of oxygen.

In the original diet C, with lard as the source of fat, no dietary destruction

TABLE II
Changes in Iodine Number and Concentration of Butter Yellow* in Different Food Mixtures

Experiment No.	Food mixtures	5th day		8th day		21st day	
		Iodine No.	Butter yellow* mg./gm. diet	Iodine No.	Butter yellow* mg./gm. diet	Iodine No.	Butter yellow* mg./gm. diet
1	"By" = A† + 16 parts linoleic acid "glyco"‡	72.0	0.15	42.8	0.09	34.3	0.07
2	"By:" 2 gm. sealed in 5 cc. tube under nitrogen	144.2	...	144.8	0.59
	"By:" 2 gm. sealed in 5 cc. tube under oxygen	140.2	0.37	129.7	0.33
	"By:" exposed to air	56.3	0.09
3	A + 16 parts arachidonic acid	176.8	0.35	104.1	0.16	68.3	0.11
	A + 16 parts linoleic acid	67.4	0.08	59.1	0.07	42.5	0.06
	A + 16 parts oleic acid	101.8	0.44	104.5	0.35	95.1	0.12
	A + 16 parts methyl oleate	98.7	0.66	98.3	0.63	91.5	0.50
	A + 16 parts palmitic acid	15.3	0.55	16.1	0.55	10.1	0.38
	A + 16 parts methyl palmitate	15.3	0.68	14.8	0.64	11.3	0.50
4	B = 16 parts linoleic acid + 0.6 mg./gm. diet of butter yellow	...	0.56	135.0	0.60
	B + 84 parts brown rice	133.1	0.56	132.1	0.50
5	B + 84 parts brown rice	134.0	0.69	133.7	0.66	134.7	0.59
	B + 84 parts white rice	123.8	0.66	125.2	0.49	64.7	0.09
6	B + 84 parts brown rice	118.7	0.61	121.1	0.66
	B + 84 parts quartz sand	86.4	0.22	52.0	0.07
	B + 84 parts sucrose	81.8	0.22	40.3	0.06
	B + 84 parts cornstarch	70.8	0.17	32.9	0.07
	B + 84 parts rice starch	58.0	0.13	37.3	0.08

* Determined colorimetrically in chloroform extract.

† Ration A had the following composition: casein 6 parts, sucrose 22, cornstarch 50, salt mixture 4, cod liver oil 2, supplemented with butter yellow. The concentration of butter yellow was maintained throughout (Experiments 1 to 6) at the level of 0.6 mg. per gm. of the complete food mixtures (i.e., per 100 parts of the mixture).

‡ The iodine number of different samples before addition to the diet varied from 124.1 to 131.8.

|| The fatty acids and esters of this experiment were purified samples prepared in the laboratory of one of us.

of butter yellow was visible *in vitro*. It can be assumed that the liberation of unsaturated fatty acids from lard in the process of digestion opens the way to decomposition of butter yellow before it reaches the liver cells. The question

why at the same time a lard diet does not entail toxic manifestations, such as are seen after administration of a diet containing linoleic acid, needs further study for its elucidation.

If these considerations are correct, the procarcinogenic effect of crisco or butter fat as well as that of rice could be explained by the preservation of butter yellow in the diet and the intestine because of a low intake of unsaturated fatty acids, in the case of butter fat or crisco, and by the presence of a stabilizer or antioxidant, in the case of rice.

According to the literature (4, 5) the production of butter yellow cancer is hindered by administration of rice bran oil obtained by extraction from rice bran with ether. This fact puts rice bran oil in the same category as lard. Furthermore it tends to corroborate the assumption that the antioxidant of rice is not fat-soluble and does not pass into the ether extract.

The metabolism of *N,N*-dimethylaminoazobenzene has hitherto been regarded as a cellular process (6). The investigations reported here throw light on a reaction which occurs without the participation of living tissue. In the light of these findings it is noteworthy that recently the destruction of hemoglobin and hemin (7) and of carotene (8) by linoleic acid has also been reported. It remains to be shown whether the conservation and destruction of butter yellow in the diet and in the intestine, as illustrated by the investigations here analyzed, exhaust all possibilities of the influence of diet on "butter yellow cancer." No such claim is presented here.

Recently great interest has been aroused by the identification of biotin as a procarcinogenic agent for butter yellow cancer (9). In the relevant experiments of du Vigneaud and his collaborators biotin was given by mouth and thus it may have acted locally in the dietary mixture or in the intestine as stabilizer for butter yellow, an effect similar to that of rice. In preliminary experiments, however, which are being continued, we have been unable to demonstrate such an effect *in vitro*.

The production of progressive anemia and leucopenia when linoleic acid is contained in the diet and their prevention by the administration of yeast recall the old hypothesis concerning the rôle ascribed to unsaturated fatty acids, mainly oleic acid, in the pathogenesis of pernicious anemia and anemia caused by tapeworm (*Bothriocephalus latus*) (10). The fact that in these previous experiments anemia could be produced by parenteral, but not by oral, administration of oleic acid has never been explained. That essential fatty acids or rather, probably, some of their oxidative break-down products, exert an injurious effect on hematopoiesis only if the diet is deficient is a fact not known hitherto and one which has become evident only in the present experiments. Further studies are needed in order to identify this deficiency which, together with the pathogenesis of "butter yellow cancer," is a good illustration of the

often recurring problem whether intoxication or deficiency (11) plays the leading part in a given dietary disturbance.

The special type of anemia and the cancer of the liver found in the present investigations were mutually exclusive. In this connection it is illuminating that in certain races of the Far East primary cancer of the liver is a common disease whereas pernicious anemia occurs very rarely (12). It should be emphasized, however, that the morphologic blood picture of pernicious anemia differs from that seen in rats fed a diet containing linoleic acid, the latter anemia being of the secondary type. This difference may be due either to the differing response of man and rat or to the differing pathogenesis.

SUMMARY

Crude linoleic acid incorporated with or without butter yellow in a synthetic diet proved to be toxic for rats. The toxic effect manifested itself in loss of weight, progressive anemia of the secondary type, leucopenia, and pediculosis. It could be neutralized preventively and therapeutically by administration of yeast. The toxicity of the diet containing linoleic acid appears to be due to oxidative break-down products of the unsaturated fatty acid.

The color of the same diet when it contained crude linoleic acid supplemented with butter yellow faded progressively in the presence of air (O_2), even at room temperature. Purified preparations of linoleic acid and, to a less degree, purified preparations of arachidonic and oleic acids have shown the same destructive effect on butter yellow *in vitro*.

Brown (unpolished) or white rice contains a stabilizer (antioxidant) for the preservation of butter yellow.

In experiments on the production of hepatoma in rats following the ingestion of butter yellow, rice on one hand and crisco or butter fat on the other hand have proved to be procarcinogenic. These results would seem to be correlated with the preservation of butter yellow in the diet and in the intestine, because of the antioxidant in rice and the low supply of unsaturated fatty acids, respectively.

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COMPARISON OF NUTRITIVE VALUE OF REFINED COCONUT OIL AND BUTTERFAT¹

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This study was undertaken to determine whether there is a difference in the general nutritive value of butterfat and coconut oil and whether diets containing 25 per cent of either of these oils will produce fatty infiltration or degeneration of the liver when fed to young, healthy, adult rats.

No reports were found in the scientific or clinical literature which indicated that there is a significant difference in the nutritive value of these fats or which indicated that they were toxic when taken in ordinary diets in normal quantities by normal individuals, in normal quantities by abnormal individuals, or in abnormal quantities by normal individuals. It has been established that the animal body has the ability to synthesize its fats from the food fats or other compounds which occur in the food.

Significant portions of mankind consume large quantities of coconut oil. Some tropical peoples use coconut oil exclusively in cooking.

Fatty degeneration of the liver can be produced by an abnormal diet in which certain amino-acid constituents (cystidine, according to Tucker and Eckstein (1938), are low or certain others (methionine) are present in increased quantities. Such fatty livers are only of clinical interest, for these diets are entirely artificial and the development of fatty livers is not dependent upon the type of fat which is fed.

In the work being reported experimental animals were given diets which were abnormal in only one respect, in that they contained about four times the normal amount of fat. This was done to accentuate any tendency toward the production of abnormalities by the coconut oil or butterfat. In all other important respects they were well balanced and adequate for the support of active growth and normal health.

¹Contribution No. 167 from the Biological Research Laboratories, Massachusetts Institute of Technology, Cambridge, Massachusetts.

EXPERIMENTAL DIET

The experimental diets used in these tests were constituted as follows:

	Coconut-oil diet	Butterfat diet
Extracted milk powder.....	72%	72%
Butterfat	25%
Coconut oil	25%
Extracted yeast	3%	3%

Roller-dried skim-milk powder was extracted twice for 24 hours with ethyl alcohol and twice for 24 hours with anhydrous ethyl ether. The brewer's yeast was extracted by the same procedure. In addition, each animal received daily an equivalent of six U.S.P. units of vitamin D in the form of Viosterol (Mead's), an equivalent of 60 U.S.P. units of vitamin A (Hickman distillate, 200,000 units per gram), and an equivalent of three milligrams of iron in the form of ferric chloride. The vitamin A and vitamin D were fed in mineral oil and the ferric chloride was mixed in the diet.

The butterfat used in these studies was purchased as butter in three local retail stores and prepared by melting and passing through filter paper in a steam-jacketed funnel.

Five one-gallon cans of refined edible 76 coconut oil were received from Durkee Famous Foods, Chicago, Illinois. It was stated by the refiners to have the following characteristics, of which the free fatty-acid content, the iodine and saponification number, and the melting point, were confirmed by our analyses.

Color, Lovibond scale.....	0.7 red
	5.0 yellow
Free fatty acid, calculated as oleic.....	0.014%
Refractive index, butyro at 45° C.....	36.9
Setting point.....	23.0°C.
Iodine value.....	8.85
Saponification number.....	256.5
Capillary melting point.....	77.0°C.
Flavor.....	Good

This oil was melted and passed through filter paper in a steam-jacketed funnel in the same manner as the butterfat.

STOCK DIET

An acceptable stock diet was fed to a third series of animals. This diet according to Russell (1932), consisted of 60 per cent ground whole wheat, 30 per cent dried whole milk, and 10 per cent meat scrap (Swift's) with the addition of sodium chloride to the extent

of two per cent of the weight of the wheat. The animals on this normal and adequate diet served as controls or standards of normality since they were litter mates to the animals used in the two experimental series.

EXPERIMENTAL PROCEDURE

Wistar strain albino rats were used in this investigation. They were placed on the experimental diets when 120 days old. Each animal was separately housed in a galvanized wire cage with raised bottom. Twelve males and 13 females were fed the coconut-oil diet, 12 males and 13 females were fed the butterfat diet, and four males and four females were fed the stock diet.

The diet was fed *ad libitum*, but the amount of food consumed by each rat was determined periodically. Distilled water was supplied at all times.

At the end of 15 days, three male and three female rats from each group were guillotined and sections of the liver, heart, lungs, kidneys, skin, subcutaneous fat, adrenals, and femur of each were taken for microscopic examination and the remaining portions of the internal organs were preserved in alcohol. The livers and the carcasses were submitted to the chemical analysis detailed below.

Similarly, at the end of 30, 60, and 90 days animals from each group were killed and studied in the same manner as those destroyed at the end of 15 days. In addition, at the end of 60 and 90 days rats (three and five, respectively) which had been on the stock diet were destroyed and submitted to the same pathological and chemical analysis.

Chemical Study of Body Tissue: The entrail-free carcasses of each group of animals were split into strips, passed through a meat grinder, and weighed. This material was then dried to constant weight at 105°C. (221°F.), pulverized in a coffee mill, placed in a Soxhlet extractor, and extracted for 18 hours with 95 per cent ethyl alcohol, then 18 hours with ethyl ether (U.S.P.). The solvent was removed from the combined extracts and the weight of the fat determined. From the data so obtained the moisture content and the fat content (alcohol-ether soluble fraction), of the body tissues were calculated.

Chemical Study of Liver Tissue: After a section of the liver of each animal was taken for pathological study the livers of each group were pooled, macerated, transferred to a watch crystal tared with one 11.0 centimeter ashless filter paper, and weighed. The samples were then dried to constant weight at 105°C., transferred quantitatively to Soxhlet extractors, and extracted for 18 hours with 95 per cent ethyl alcohol, then for 18 hours with ethyl ether (U.S.P.). The combined extracts were evaporated to dryness and weighed.

From the data the moisture content and the fat content (alcohol-ether soluble fraction) of the livers were calculated.

Determination of True Lipids in Liver and Body Tissues: The true lipid (petroleum-ether soluble) content of the liver and body tissues of the rats which had been on the three diets for 60 and 90 days was determined on the substance obtained by extracting the alcohol and ether extracts with petroleum ether.

EXPERIMENTAL RESULTS

Weight Increase: Growth of the animals during the test is shown by the following summary tabulation:

Days on diet	n	Body weight in grams				Total increase
		15	30	60	90	
Rats (25) on butterfat diet.....	208	217	229	228	240	32
Rats (25) on coconut-oil diet.....	208	217	232	244	258	50
Rats (5) on stock diet.....	210	224	235	246	274	64

These results indicate that during the first 30 days of the test, both groups of animals grew at the same rate, while during the next 60 days the animals on the coconut-oil diet grew much more rapidly than those on the butterfat diet. This increase in weight was due to an increase in body tissue, not adipose tissue, for subsequent data will show that the body and liver tissue of the group fed the coconut-oil diet was no higher in fat or lipid content than that of the group fed the butterfat diet.

Food Consumption: The average amount of food consumed each day by the animals during the test period is shown by the following tabulation:

Group	Diet	Average weight of food consumed daily by each animal during			
		15 days	30 days	60 days	90 days
1	Butterfat.....	8.9 gms.	11.8 gms.	10.9 gms.	11.3 gms.
2	Coconut oil.....	7.8 gms.	11.0 gms.	10.7 gms.	11.1 gms.

The data indicate that throughout the test the animals fed the butterfat diet consumed a slightly larger amount of diet. The superior weight increase of the group fed the coconut oil was not due to greater feed consumption.

Results of Chemical Analysis of Body Tissues: Both the butterfat diet and the coconut-oil diet have a tendency to increase the fat (alcohol and ether-soluble fraction) content of the body tissues, the increase being essentially the same in both groups (Table 1).

The petroleum-ether soluble fraction (true lipid) of the body-tissue lipids was essentially the same at the end of 90 days for both

experimental groups and was about 25 per cent higher than that of the group fed the stock ration.

These chemical results indicate that a diet containing 25 per cent butterfat or 25 per cent coconut oil produces a slight fatty infiltration of the body tissues of rats which is measurable by chemical analysis.

TABLE 1
Summary of Chemical Data

BODY TISSUES						
Group	Diet	Days on diet	Moisture	Fat ¹		True lipid ²
				Wet	Dry	
			<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>
1	Butterfat	15	65.9	10.3	30.7
2	Coconut oil	15	67.9	9.3	29.7
1	Butterfat	30	65.9	11.7	34.2
2	Coconut oil	30	67.2	11.6	35.2
1	Butterfat	60	64.3	12.5	34.9	24.0
2	Coconut oil	60	64.1	13.3	36.9	24.8
3	Stock	60	67.1	8.4	25.8	19.7
1	Butterfat	90	64.7	13.9	39.3	28.2
2	Coconut oil	90	64.9	13.7	38.8	27.4
3	Stock	90	61.9	11.3	29.6	23.2
LIVER TISSUES						
1	Butterfat	15	72.0	6.0	21.3
2	Coconut oil	15	73.0	5.7	21.1
1	Butterfat	30	71.6	6.5	22.3
2	Coconut oil	30	71.5	6.2	21.7
1	Butterfat	60	70.6	6.9	23.7	18.8
2	Coconut oil	60	70.4	7.2	24.0	18.5
3	Stock	60	70.8	4.8	16.5	13.6
1	Butterfat	90	70.5	7.3	24.6	19.3
2	Coconut oil	90	69.9	8.0	26.4	20.4
3	Stock	90	70.6	6.8	23.0	17.0

¹ Fat = alcohol and ether extract. ² True lipid = petroleum-ether soluble lipid.

Results of Chemical Analysis of Liver Tissues: The data indicate that the fat content of the livers of animals on the butterfat and the coconut-oil diets progressively increased during the test period (Table 1). This tendency was essentially the same in both groups of animals.

The true lipid content of the liver tissue of both groups of experimental animals was essentially the same and 20 to 30 per cent higher than the true lipid content of the liver tissue of the group fed the stock ration.

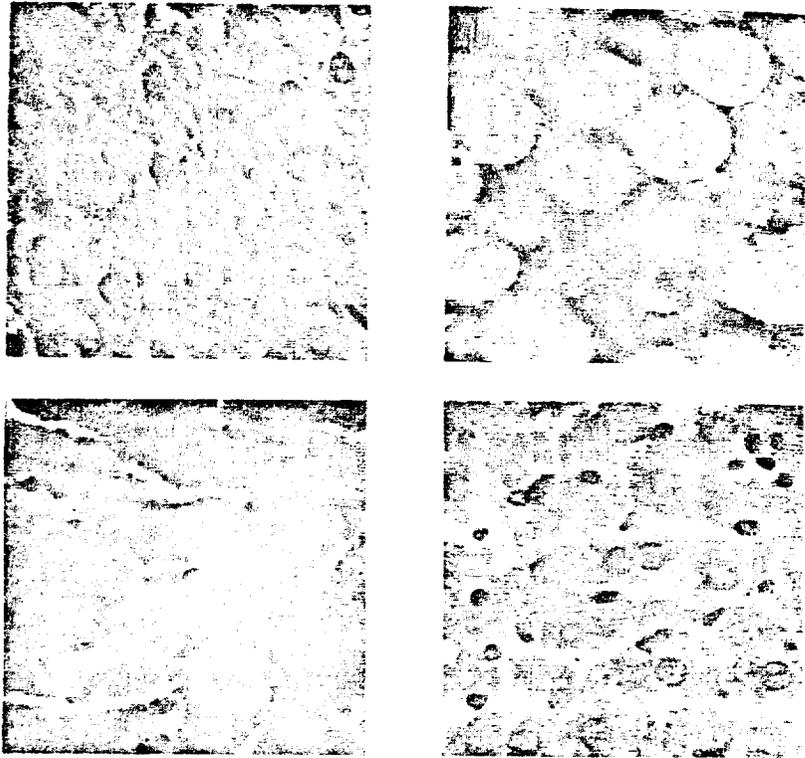


FIG. 1. Photomicrographs (50x) of typical liver sections stained with hematoxylin and eosin. White vacuoles represent fat droplets.
 Upper left: from normal stock diet.
 Upper right: example of true fatty infiltration, animal not in this series of experiments.
 Lower left: from rat fed coconut oil diet.
 Lower right: from rat fed butterfat diet.

Milk-powder diets, containing 25 per cent coconut oil or 25 per cent butterfat, therefore produce a measurable amount of fatty infiltration of the liver tissue of rats.

PATHOLOGICAL FINDINGS

The livers of all animals were studied histologically using Scharrlach R and hematoxylin and eosin stains. There was no significant variation within groups, between groups, or between series, nor was there any evidence of pathological fatty infiltration in any animal. There was evidence of a very slight amount of infiltration of fat into the cytoplasm of the liver tissues (Fig. 1).

There were no pathological findings on histologic examination of the lung, heart, kidney, spleen, gastrointestinal tract, skin, muscle,

subcutaneous tissue, and bone (femur) of any of the animals which had been on the experimental or stock diets for 90 days. Thus, histologically, the chemical observation that these diets produced a mild tissue infiltration was confirmed. Furthermore, these results indicate that coconut oil and butterfat are equally harmless to rats, even when they constitute rather high proportions of the diet.

SUMMARY AND CONCLUSIONS

Experimental rats were maintained on a diet consisting of extracted skim-milk powder (72 per cent), extracted brewer's yeast (three per cent), and refined coconut oil (25 per cent) or butterfat (25 per cent), supplemented with vitamin A, vitamin D, and iron. These diets were abnormal in only one respect, that is, they contained an abnormally large proportion of fat. The animals were observed as to the effects of these two diets on weight increase and food consumption. Groups were guillotined after 15, 30, 60, and 90 days on the diets, and body tissues were studied histologically. Results were compared with those on rats maintained on a standard stock ration which served as control.

Animals on the butterfat diet consumed a slightly larger, but possibly insignificant, amount of diet but increased in weight much less rapidly than the animals on the coconut-oil diet.

The superior weight increase of the rats on the coconut-oil diet was not adipose tissue, for the body and liver tissues of the groups contained essentially the same amount of fat (alcohol-ether extract) and true lipid (petroleum-ether extract of the alcohol-ether extract). The investigation is being extended to determine whether the weight increase was due to increase in muscle tissue.

The animals on both the butterfat and the coconut-oil diets developed a slight fatty infiltration of the body and liver cytoplasm. This was shown by increased amounts of fat and of true lipids and by histological examination. This fatty infiltration was equally intense in the coconut-oil and butterfat groups.

There was no evidence of pathological tissue changes in any animal in any group.

These results indicate that butterfat and coconut oil, even when fed at rather high levels in a complete diet, are equally harmless to rats and presumably to man.

ACKNOWLEDGMENT

Dr. Sidney Farber, pathologist, Infants' and Children's Hospital, Boston, prepared, examined, and interpreted all of the histological sections. We greatly appreciate his cooperation.

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Tumor Promoting Action of Fatty Acids in the Skin of Mice

by Paul Holsti

In their investigations of the action of various solvents on the skin tumor formation in mice, Twort and Twort (1) have found that oleic acid (following previous repeated carcinogen treatment) applied 5 times per week on the skin of the test animals, numerically promoted the tumor formation. According to the understanding of the biphasic nature of the experimental skin carcinogenesis it was customary to test the tumor promoting action of a given substance by a technique by which the substance concerned, after a single suboptimal carcinogen dose (initiation), was applied twice per week to the skin of the test animals. Under such conditions a tumor promoting action could be observed neither by the oleic acid nor by other oils tested in the same way (2).

On the other hand, certain non-ionizable detergents in which the molecular structure is comprised of different fatty acids as components, have been proven to be extraordinarily strong tumor promoters in the skin of mice (3). Previously performed investigations with these substances have further shown that the application frequency of the tumor promoters has a great significance for the appearance of the tumors, as hitherto had been assumed.

For these reasons the possible tumor promoting ability of several biologically important fatty acids was tested. All of the mice used for the tests were of the same unnamed white stock. Each group included 44 animals (22 male and 22 female mice). The initiation took place by a single treatment of the concerned spot of skin with 9,10-dimethyl-1,2-benzanthracene (DMBA) from the firm L. Light and Co., Ltd., Colnbrook, England (0.3% dissolved in colorless, non-fluorescent paraffin oil). The fatty acids to be tested were applied to the skin once daily 6 days per week. The non-fluids (stearic acid from the firm E. Merck AG., Darmstadt, Germany, and palmitic and lauric acid from the firm Eastman Kodak Co., Rochester, USA) were applied in 20% luke warm chloroform solution; the oleic acid (from the firm May and Baker, Dagenham, England) in addition was applied to a special group undiluted. Special care was taken to prevent a carcinogen contamination.

Table 1 gives the maximum tumor incidence during the test period of 24 weeks.

In the group with undiluted oleic acid the first tumor appeared 10 weeks, in the group with 20% oleic and 20% lauric acid 14 and 15 weeks, respectively, after the beginning of the treatment. The difficulty to dissolve stearic acid and palmitic acid separated from the chloroform solution upon contact with the skin and were evidently unable to infiltrate the skin to a degree worth mentioning.

The maximum tumor incidence in all tumor groups was greater among the male animals. Thus, for example, in the group with undiluted oleic acid the maximum tumor incidence for the male mice was 73% and for the females 43%. Also in a series carried out as a comparison, with the non-ionizable detergent Tween 60 as the tumor promoter, the maximum tumor incidence was greater with the male (64%) than with the female (41%) animals. In the earlier works with detergents male mice were used exclusively (4). During the work on this manuscript it became known to us that a sex difference is also being observed elsewhere (5).

The investigations were carried out. The tumor promoting ability of 4 different acids of the fatty acid series and also that of some acids not belonging to it was investigated. A detailed report of the results will appear later.

With support of the Rockefeller Foundation.

Table 1

Primary treatment (initiation)	Secondary treatment (tumor promotion)	Max. tumor incidence (in %)
0.3% DMBA	none	0
none	undiluted oleic acid	0
0.3% DMBA	undiluted oleic acid	56
0.3% DMBA	20% oleic acid	32
0.3% DMBA	20% lauric acid	31
0.3% DMBA	20% stearic acid	0
0.3% DMBA	20% palmitic acid	0
0.3% DMBA	chloroform	0

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FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI,
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TUMOR PROMOTING EFFECTS OF SOME LONG CHAIN FATTY ACIDS IN EXPERIMENTAL SKIN CARCINOGENESIS IN THE MOUSE¹

By

PAUL HOLSTI²

Received 30.1.59

While the enhancing effect of high fat diets in experimental skin carcinogenesis is a well established fact (for a review of these aspects see ref. 4 and 13), the mode of action of dietary fat and the effects of local application on the skin of fatty acids during experimental skin carcinogenesis have been variously interpreted. It was early established (14) that several substances belonging to the group of lipids, used as solvents for the carcinogen influenced in various ways the development of tumors in the skin. In connection with some of these investigations it was also observed that oleic acid applied five times weekly after repeated (thirty) applications of the carcinogen had a "sensitizing" effect enhancing the development of local cutaneous tumors (14). However, the mortality in these experiments was very high, only 8 of 100 mice surviving 35 weeks in the oleic acid series, and, as a result of the prolonged treatment with the carcinogen, several tumors developed also in the series treated only with the carcinogen. The picture emerging from these experiments concerning the effect of locally applied fatty substances, especially fatty acids, was thus complex and the interpretation of the results not clear.

When, as a result of later investigations, the process of experimental skin carcinogenesis was more sharply divided into different stages (e.g., according to the now most commonly used nomenclature "initiation" and "promotion") and the experiments were planned accordingly, no "promoting" effect could be demonstrated by the use of oleic acid applied according to current usage twice weekly after a single application of the carcinogen (11). The earlier observed enhancing effect of oleic acid (*vide supra*) was apparently generally considered to be connected with the preceding frequent application of the carcinogen only.

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It was then shown that certain non-ionic lipophilic-hydrophilic compounds which are polyoxyethylene sorbitan esters, or sorbitan esters, of higher fatty acids (*e.g.*, some of the Tweens and Spans) were under certain conditions potent tumor promoters for the mouse skin (6, 7, 9, 10). With the aid of these compounds (technical as well as synthesized in the laboratory) the importance of the frequency with which the promoter is applied was demonstrated and the tumor promoting effect was shown to be correlated to the degree and duration of the induced epidermal hyperplasia (1, 8, 10).

Considering the results of the various experiments with oleic acid (11, 14) against the background of this established importance of the frequency with which the promoter is applied, the author's attention was drawn to the possibility that some of the fatty acids as such, when applied more frequently than twice a week, might exhibit tumor promoting properties. The present paper reports the results of experiments where some long chain fatty acids present in the molecule of the above mentioned lipophilic-hydrophilic compounds were tested for tumor promoting activity on the mouse skin by use of the frequent application technique.

MATERIAL AND METHODS

The experimental animals were 4 to 8 months old male and female white mice of an anonymous non-inbred strain reared in this laboratory. The number and sex of the animals in each series are listed at the respective places in the text. As in the previous experiments (10), the usual stock diet of the laboratory, which had proven sufficient for the rearing of the animals, was used and water was given *ad libitum*.

The site of treatment was an area (2.0 x 1.5 cm) on the animals back, dextrad to the median line. The hair was cut at regular intervals from this area with scissors.

Initiation was accomplished by a single local application of a 0.3 per cent solution of 9,10-dimethyl-1,2-benzanthracene (DMBA), dissolved in colorless, non-fluorescent liquid paraffin. Special care was taken to avoid later contamination with any carcinogen.

The investigated fatty acids were: Oleic acid (May & Baker, Ltd., England), stearic acid (E. Merck A. G., Germany) and palmitic and lauric acid (Eastman Kodak Co., USA). Of these, oleic acid was used as promoter both undiluted and as a 20 per cent solution in chloroform (Pharmacopoea Fennica), while lauric, stearic and palmitic acids were used as promoters only as 20 per cent solutions dissolved in chloroform. The two last mentioned fatty acids are less soluble in chloroform and their solutions were before the application heated in order to clarify them. Since one of the most potent of the technical non-ionic lipophilic-hydrophilic promoters previously mentioned, Tween 60, has been found to contain both stearic and palmitic acid (8), a mixture of these acids (6:4) was used in one series as a 20 per cent solution dissolved in chloroform.

Although fresh solutions were frequently made, the evaporation of the chloroform during the period of use increased the concentration of the dissolved fatty acids up to about 40 per cent at the time of exchange of the bottles.

Unless otherwise stated, the investigated compounds were applied to the initiated area of the mouse skin once daily (six times a week) with a No. 2 water color brush. This treatment was started 24 hours after the initiation, with the exception of series No. 9, in which an interval of 30 days was held between initiation and commencement of the treatment with oleic acid. The tumors appearing in the treated area (none occurred at other sites) were recorded weekly and the incidence of tumors was calculated from the number of experimental animals surviving at the time of appearance of the first tumor in the series.

RESULTS

Tumor Promoting Effect of the Investigated Fatty Acids when Applied Once Daily.

The results of the tumor promoting experiments up to the 31st week are listed in Table 1. Each series in this experiment comprised 44 animals (22 male and 22 female mice).

TABLE I
Tumor Promoting Effect of Long Chain Fatty Acids Applied Six Times a Week. Initiation by a Single Application of 0.3 per Cent DMBA in Liquid Paraffin. Chloroform Used as Solvent in Series 4-8. Duration of Experiment 31 Weeks.

No. of Series	Test Compound	First Tumor		Maximum Incidence of Tumors		No. of Mice Alive		
		Time (Wks.)	No. of Mice Alive	Time (Wks.)	Tumors per Tumor-Bearing Mouse	Per cent	At 25 Weeks	At 31 weeks
1	None				0	0	41	36
2	Chloroform				0	0	40	29
3	Oleic acid, undil.	10	14	25	78/27	61	34	28
4	Oleic acid, 20%*	11	12	23	25/13	31	37	21
5	Stearic acid, 20%*				0	0	38	25
6	Stearic + palmitic acids				0	0	35	24
7	Palmitic acid, 20%*				0	0	40	25
8	Lauric acid, 20%*	15	12	28	30/14	33	38	29

* Starting concentration (see Material and Methods).

As seen from the table, undiluted oleic acid as well as oleic and lauric acids dissolved in chloroform exhibit distinct tumor promoting properties. Tumors appeared first in the series in which oleic acid was used undiluted. In the two series (No. 4 and No. 8) in which oleic and lauric acids were dissolved in chloroform the time for the first appearance of tumors, the maximum incidence of tumors and the number of tumors per tumor bearing animal were fairly similar, while the point of maximum incidence of tumors was reached somewhat earlier in the oleic acid series (No. 4). In series No. 5, 6 and 7, where the poorly soluble stearic and palmitic acids were used, the skin of the animals was shortly after each treatment found to be covered with a white layer, the chloroform having apparently evaporated before the fatty acids had been able to penetrate into the skin.

In all the series in which chloroform was used as solvent the mice appeared transiently intoxicated at the end of each treatment. This, however, was the case also in the control series, where only chloroform was used as secondary treatment (No. 2) and where no tumors appeared.

Compound Controls.

Four experimental series were carried out without foregoing initiation: Oleic acid undiluted twice daily, oleic acid once daily as a 40 per cent solution in chloroform, and lauric acid once daily as 40 per cent and 20 per cent solutions in chloroform. The three last mentioned series comprised 30 animals each (15 of either sex) and in these series 26 to 30 animals were still alive in the 31st week. The series in which oleic acid was used undiluted twice daily comprised 44 animals (22 of either sex) and the survivors in this series (No. 12) are seen in Table 2. The compounds were applied in the same manner as in series No. 1-8. Oleic acid was thus tested without initiation on a total of 74 animals and lauric acid on 60 animals. In none of these series did any tumors appear during the period of observation (31 weeks).

Influence of Time Interval Between Initiation and Promotion.

Since in the preceding experiments the time interval between initiation and promotion was only 24 hours, it might be argued that the action of, for instance, oleic acid depended on the known enhancing effect of oils when applied *during the period of carcinogen administration* (13). Therefore, in a later additional series an interval of 30 days was held between the initiation with a single local application of 0.3 per cent DMBA in paraffin and the treatment with undiluted oleic acid (once daily). In this series (No. 9), which comprised 30 animals (15 of either sex), tumors developed in a manner similar to that in the series where the interval was only 24 hours, and the maximum tumor incidence so far (33 per cent in 19 weeks) is the same in the two series.

Influence of Frequency of Application of Oleic Acid.

As stated above, one impetus to the present investigation was given by the results obtained in earlier works in which, in addition to other differences in technique, the frequency with which oleic acid was applied varied. It was therefore necessary to investigate if the effect of oleic acid was linked to the frequency of application, as has been shown to be the case with the non-ionic lipophilic-hydrophilic promoters (10). At the same time these series were a control assuring that the positive promoting effect of oleic acid, demonstrated in the present work, did not depend on a special sensitivity of the used mouse strain. Each series in this experiment comprised 44 animals (22 males and 22 females). Initiation was as previously. Treatment with oleic acid was started 24 hours after the initiation. Thus in the series where oleic acid was used twice weekly the first application was carried out 24 hours after the initiation. The experiment lasted 25 weeks. Fig. 1 shows the development of tumors when oleic acid was used as promoter twice weekly, once daily, and twice daily, respectively. The results appear from Table 2.

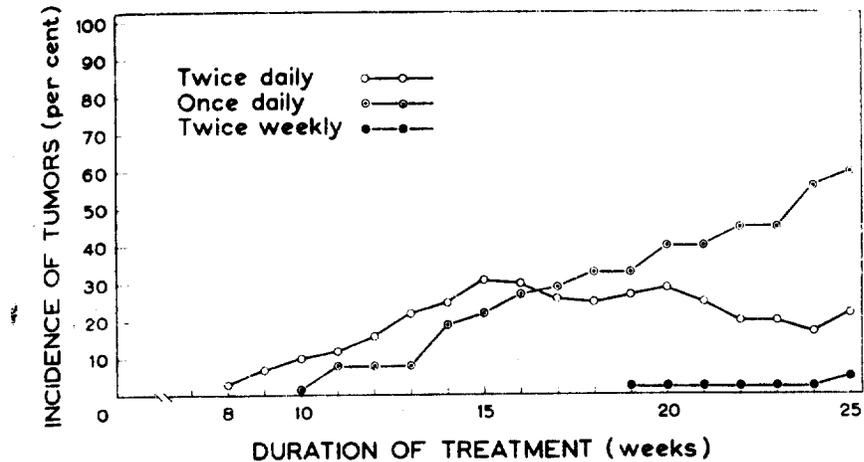


Fig. 1.

Development of Incidence of Local Skin Tumors in Series where Oleic Acid Was Used as Promoter Twice Daily, Once Daily and Twice Weekly, Respectively. Initiation with a Single Local Application of 0.3 per cent DMBA in Liquid Paraffin.

As seen from Table 2 and Fig. 1, the frequency with which oleic acid is applied as a promoter decisively influences the development of tumors.

TABLE 2

Tumor Promoting Effect of Oleic Acid Applied with Various Frequencies. Initiation in Series No. 10, 3 and 11 with a Single Application of 0.3 per Cent DMBA in Liquid Paraffin. In Series No. 12 no Initiation. Duration of Experiment 25 Weeks.

No. of Series	Test Compound	First Tumor		Maximum Incidence of Tumors			No. of Mice Alive	
		Time (Wks.)	No. of Mice Alive	Time (Wks.)	Tumors per Tumor-Bearing Mouse	Per cent	At 15 Weeks	At 25 Weeks
10	Oleic acid twice daily	8	40	15	16/13	33	40	16
3	Oleic acid once daily	10	44	25	78/27	61	42	34
11	Oleic acid twice weekly ...	19	40	25	2/2	5	41	38
12	Oleic acid twice daily	—	—	—	0	0	38	7

The more frequently oleic acid was used, the earlier the tumors developed and the more steep was the increase in the incidence of tumors. However, oleic acid applied twice daily had a fairly strong irritative effect on the skin and the general condition of the animals in these series (No. 10 and 12) rapidly became poorer, several of the animals dying especially between the 15th and 25th weeks. During this period

the incidence of tumors (Fig. 1) in the series where oleic acid was used twice daily dropped below that of the series where oleic acid was applied once daily.

Influence of Sex of Animals.

Table 3 shows the results of the tumor promoting experiments in the positive series when distributed according to the sex of the experimental animals.

TABLE 3
Influence of Sex of Experimental Animals on the Tumor Promoting Effect of Fatty Acids. Initiation, Solvent and Duration of Experiment as in Table 1.

No. of Series	Sex	Test Compound	Maximum Incidence of Tumors			
			First Tumors Time (Weeks)	Time (Weeks)	Tumors per Tumor-Bearing Mouse	Per cent
3 a	♂	Oleic acid undiluted	10	25	58.17	77
b	♀	once daily	14	29	23.12	57
4 a	♂	Oleic acid	17	23	12.6	33
b	♀	20%	14	21	11.7	32
9 a	♂	Lauric acid	15	22	13.9	45
b	♀	20%	19	24	6.5	23
10 a	♂	Oleic acid	9	15	7.6	33
b	♀	twice daily	8	15	9.7	33

Although in the series where oleic acid was used undiluted once daily and in the series with lauric acid the tumors appeared earlier and the tumor incidence reached a clearly higher level among the males, the difference between tumor incidences in the other series were negligible.

Nature of Induced Tumors.

On histological examination all the induced tumors were found to be typical papillomas. No histologically malignant tumors were found.

DISCUSSION

When oleic acid was used as a tumor promoter twice weekly after Initiation (Series No. 11 in Table 2) only one tumor was observed among 40 animals during the first 20 weeks of the experiment. This result may be regarded as comparable to the results of earlier investigations (11) where in experiments lasting 20 weeks no tumor was found among 10 animals when oleic acid was applied twice weekly. The other results of the present work, however, clearly show that oleic acid is a potent tumor promoter for mouse skin when applied with sufficient frequency (*e.g.*, once or twice daily). This also confirms the results obtained in experiments with certain of the Tweens (10) regarding the

importance of the frequency with which the tumor promoter is applied. That the effect of oleic acid is a true promoting effect, not identical with the enhancing effect of oils when applied during the period of carcinogen administration, is shown by the results in the series (No. 9) in which the treatment with oleic acid was started after an interval of 30 days from the carcinogen application.

In the present experiments distinct tumor promoting properties were exhibited also by lauric acid (in itself solid) when dissolved in chloroform. While chloroform alone under certain conditions has been shown to enhance tumor formation (2), this appears to have played no definite role under the conditions of the present experiment, since no tumors were observed in the control series when chloroform alone was used as secondary treatment with the same frequency as the investigated fatty acids. These two biologically important long chain fatty acids, which are also present in the molecule of some Tweens, thus reveal themselves as potent tumor promoters for the mouse skin even when used as such. The negative findings with, for instance, stearic acid (which is included in the molecule of one of the most potent of the Tweens, (Tween 60), conceivably may to some extent depend on its poor solubility. The white layer which the precipitated stearic acid formed on the mouse skin already a short time after the application not only was a sign of the poor penetration into the skin of this acid but may also protect the skin from the effects of the following applications of the promoter. It is, of course, possible that still other factors contribute to the negative effects obtained with this acid. The tumor promoting effect of a given substance, such as a fatty acid complex, measured in the skin is, of course, a product of several factors, including among others the actual promoting effect of the substance, its solubility and transportability.

The present experiments were designed to test a possible tumor promoting effect of the investigated fatty acids, and in the control series where oleic and lauric acids were employed without any foregoing initiation no tumors were observed. This does naturally not preclude that these acids in a more prolonged experiment (and applied with sufficient frequency) might perhaps give rise to some tumors even without initiation, as has been shown to be the case with some of the Tweens (7).

It has earlier been pointed out that Tween 60 as a promoter gives a somewhat higher tumor incidence in male mice as compared to female mice (5). In the present experiment this was the case also when undiluted oleic acid was used once daily and when lauric acid dissolved in chloroform was used. However, in the other series the differences between the sexes were negligible at the 31 week level.

The benign nature of the induced tumors is in accordance with results obtained with other promoters when 9,10-dimethyl-1,2-benzanthracene is used as initiator in the manner employed in this work (1).

It is interesting to note that some simple fatty acid esters as well as

Some of the 'Tweens' seem to have a more general growth enhancing effect, acting for instance on plants in a way somewhat similar to the plant growth substances (3, 12). It is further intriguing that while the plant growth substances exert their action principally on the elongation phase of growth, characterized by intensive water uptake, one of the most conspicuous morphological features in the reaction of the skin to the promoters containing fatty acids is the prominent inter- and intracellular edema (1).

At present a great number of fatty acids, fatty alcohols and related substances are being tested for their action on the mouse skin. The results of these experiments will be reported later.

SUMMARY

Some long chain fatty acids which form a part of the molecule of the lipophilic-hydrophilic tumor promoters (certain Tweens and Spans) have been tested for tumor promoting activity on the skin of mice, following initiation with a single local application of 0.3 per cent 9,10-dimethyl-1,2-benzanthracene in liquid paraffin.

Undiluted oleic acid, as well as oleic and lauric acids dissolved in chloroform, were shown to exhibit significant tumor promoting properties when applied to the skin once daily (six times a week).

The promoting action of oleic acid was shown to be linked to the frequency of application, thus confirming the results obtained in the investigations with Tween 60 on the influence of the frequency with which the promoter is applied.

When the less soluble stearic and palmitic acids were used (dissolved in chloroform) the skin became covered with a white layer of precipitated acid after each treatment. No tumors developed in these series.

In two of the series (undiluted oleic acid and lauric acid once daily) the male mice showed a significantly higher incidence of tumors, while in the other series no sex difference could be observed.

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FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

TUMOR-PROMOTING EFFECTS IN MOUSE SKIN

COMPARISON BETWEEN OLEIC ACID, SORBITAN MONO-OLEATE (SPAN 80),
AND POLYOXYETHYLENE SORBITAN MONO-OLEATE (TWEEN 80)

by

PAUL HOLSTI

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Following the establishment of the tumor promoting effect of the non-ionic lipophilic-hydrophilic Tweens and Spans (4, 6), and the experiments carried out with these compounds on the mechanism of tumor promotion (1, 3, 5, 7, 8), it was shown that some of the long chain fatty acids contained in the molecule of these compounds were able to exhibit tumor promoting properties in the mouse skin even when used as free acids (2). A comparison of the promoting activity of a Tween, a Span and their respective fatty acid was therefore undertaken under as identical conditions as possible. Because oleic acid offers special advantages, being a liquid, over the other fatty acids of the various Tweens and Spans, this acid and a Tween and a Span containing it were chosen as subjects of the investigation. The purpose of the experiment was exclusively to compare the tumor promoting effect of the chosen compounds when applied on a previously initiated area of mouse skin. For the effects of these compounds when used without foregoing initiation see ref. 2 and 4.

MATERIAL AND METHODS

The experimental animals were male and female mice of an anonymous outbred strain, raised (and temporarily in use) in this

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laboratory, and not identical with the strain used in the previous experiments with Tweens and Spans (4, 6). The animals were 4 to 6 months old at the commencement of the experiment and with respect to age and sex exactly equally distributed among the various groups, each of which comprised 44 animals (22 male and 22 female mice). The same stock diet as in the previous experiments (2, 4) was used and water was given ad libitum.

Initiation was accomplished in all the series on the same day and by the same person, by a single, local application of a 0.3 per cent solution of 9,10-dimethyl-1,2-benzanthracene (DMBA) in colorless, non-fluorescent liquid paraffin. The investigated compounds were: Tween 80 (polyoxyethylene sorbitan monooleate) and Span 80 (sorbitan monooleate) from Atlas Powder Co., USA, and oleic acid from May & Baker Ltd., England. The treatment with these compounds was started 24 hours after the initiation and the compounds were applied with a No. 2 water color brush by the same person once daily (six times a week) to the previously initiated area of skin on the animals back. The tumors were recorded once weekly and the incidence of tumors was calculated from the number of animals surviving at the time of appearance of the first tumor in the series concerned.

RESULTS

The development of skin tumors in the various groups is pictured in Fig. 1, while the number of tumors and of surviving animals are listed in Table 1. The experiment lasted 24 weeks.

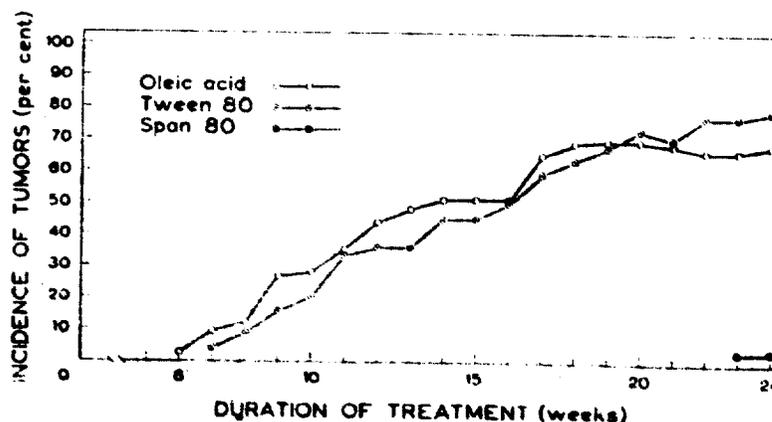


Fig. 1. — Development of local skin tumors with daily (6 times a week) treatment with oleic acid, Tween 80 and Span 80 after initiation with a single local application of 0.3 per cent DMBA in liquid paraffin.

TABLE 1

Test Compound	First Tumor		Maximum Incidence of Tumors		No. of Mice Alive at 24 Weeks
	Time (Weeks)	No. of Mice Alive	Time (Weeks)	Tumors per Tumor-Bearing Mouse	
Oleic acid	6	43	19	131/30	36
Tween 80	7	44	24	143/35	43
Span 80	23	38	23	1/1	38

As seen from Fig. 1, the incidence of skin tumors in the series treated with oleic acid developed in a manner closely similar to that in the series treated with Tween 80.

The number of tumors per tumor bearing animal (Table 1) in these two series also largely corresponds, while in the series treated with Span 80 only one tumor was observed.

DISCUSSION

Most of the fatty acids contained in the molecule of the various Tweens and Spans (stearic, palmitic and lauric acids) are solid and practically insoluble in water. Therefore a direct comparison of the effects on the skin of these acids and their respective Tween- and Span-type products is not feasible. In this respect oleic acid, being a liquid, is an exception and makes such a comparison possible. When, in the present work, undiluted oleic acid and polyoxyethylene sorbitan monooleate (Tween 80) were used as promoters under identical experimental conditions, tumors developed according to a closely similar pattern in the two series. It should be borne in mind, however, that the molecule of Tween 80 contains only about 21.5 per cent oleic acid (4). The incorporation of oleic acid into the total molecule of Tween 80 probably makes it more soluble and more readily transportable. This does not exclude the possibility that the total molecule of the Tweens may still have other important properties that influence the development of tumors. It is also to be noted that Span 80 (sorbitan monooleate) in the present investigation, as in the previous experiments (4), exhibits only feeble tumor promoting activity. The actual incidence of tumors in the

present work should not be compared with that in the earlier investigation with the Tweens and Spans (4), since a different strain of mice was used.

SUMMARY

The tumor promoting activity in the mouse skin of oleic acid, sorbitan mono-oleate (Span 80) and polyoxyethylene sorbitan monooleate (Tween 80) was compared.

Under the conditions of the present experiment and during the period of observation of 24 weeks the development of local skin tumors followed a practically identical course in the series treated with oleic acid and that treated with Tween 80. In agreement with the results of previous investigations, Span 80 exhibited only very weak tumor promoting activity.

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THE NATURE AND MECHANISM OF ACTION OF
UNCOUPLING AGENTS PRESENT IN MITOCHROME PREPARATIONS

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SUMMARY

1. The uncoupling activity of mitochrome preparations was not affected by addition of $\text{Na}_2\text{S}_2\text{O}_4$, $\text{K}_3\text{Fe}(\text{CN})_6$, CO or $10^{-4} M$ KCN, all of which bring about changes in the absorption spectrum of mitochrome.
2. The uncoupling activity was also not affected by precipitation of the protein by heating the solution or adding trichloroacetic acid.
3. The substance(s) responsible for the uncoupling activity could be completely extracted by organic solvents; the mitochrome remained in the water layer.
4. Purified preparations of cytochrome ($a + a_3$) or cytochrome *b* changed into mitochrome on standing at 4° for a few days. Simultaneously, there was an increase in the amount of uncoupling material which could be extracted from these preparations by organic solvents.
5. The uncoupling activity of an iso-octane extract of mitochrome resides in the unsaturated fatty acids, chiefly oleic and linoleic acids, which it contains.

INTRODUCTION

In 1952 LARDY¹ suggested that one of the mechanisms of the control of cell metabolism might be the presence in the cell of substances which uncouple oxidative phosphorylation. LARDY's idea could be extended by postulating that the uncoupling substance is not always present in an active form, but is liberated from a precursor under certain conditions. Indeed, freshly prepared liver mitochondria are not appreciably uncoupled, but on standing at room temperature a suspension develops many of the characteristics of freshly prepared mitochondria to which a typical uncoupling agent such as dinitrophenol is added, namely a decrease of the P:O ratio and of the P_i -ATP exchange reaction, and an increased ATPase activity. At the same time, structural damage is observed, particularly to the mitochondrial membranes. This suggests that during the "ageing" of the mitochondria, an uncoupling agent is produced, possibly from the mitochondrial membranes.

An uncoupling agent was first isolated from disintegrated, aged mitochondria

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i , inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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by POLIS AND SHMUKLER² and PULLMAN AND RACKER³. The purified compound² was called mitochrome because it had the properties of a haemoprotein. Experiments will be reported in this paper confirming the uncoupling action of the mitochrome preparation. However, we have found that this cannot be ascribed to the haemoprotein itself but to components, extractable by organic solvents, present in the mitochrome preparations. Preparations of the cytochromes ($a + a_3$) and b , which yielded mitochrome on ageing, were also found to contain the uncoupling agents. A preliminary report of some of these findings has appeared⁴.

METHODS

Reagents

The ATP and Tris were obtained from Sigma Chemical Co. $H_3^{32}PO_4$ was purchased from Philips Roxane (Holland), bovine serum albumin from Armour, and isooctane from Brocades-Stheeman & Pharmacia. EDTA, ethanolamine, sucrose, KCl, 2,4-dinitrophenol and most of the other chemicals used were Analar Reagent supplied by British Drug Houses, Ltd. Hexokinase was prepared from yeast as described by BERGER *et al.*⁵ up to step 5. The water used to make up the solutions was double (glass) distilled.

Preparations

Rat-liver mitochondria were isolated in 0.25 M sucrose at 0–2° by the method of HOGEBOOM⁶, as described by MYERS AND SLATER⁷.

Mitochrome was prepared from whole beef liver according to the method of POLIS AND SHMUKLER².

Cytochrome (a + a₃) and cytochrome b preparations were made as described by ELLIOTT, HÜLSMANN AND SLATER⁸.

Extracts in organic solvents of mitochrome or of cytochrome preparations were prepared by shaking 1 volume of the preparation with 3 volumes of the organic solvent at $20 \pm 2^\circ$ for 2 min (or for 1 min when the extraction was carried out twice). After centrifugation the organic layer was removed (or when an organic solvent was used which mixed with the water layer, the entire clear supernatant was removed), and the latter evaporated to dryness on a water bath, under a stream of nitrogen. The residue thus obtained was dissolved in 96% ethanol.

Methods

ATPase activities were measured as described by MYERS AND SLATER⁷. 0.05 ml of a freshly prepared liver mitochondria preparation (0.5–1.0 mg protein) was added to an incubation medium of 1.45 ml containing, in addition to the reagents to be tested, 0.002 M ATP, 0.075 M KCl, 0.0015 M $MgCl_2$, 0.0005 M EDTA, 0.05 M sucrose and 0.05 M Tris-acetate buffer⁷ or of Tris-ammonium-acetate buffer⁷ of the desired pH. The reaction was carried out at room temperature ($20 \pm 2^\circ$). After 15 min incubation the reaction was stopped by the addition of 1.5 ml 10% trichloroacetic acid. The tubes were centrifuged at 3000 rev./min for 5 min and phosphorus determined in the clear supernatant fluid by the method of FISKE AND SUBBAROW as modified by SUMNER⁹.

Oxidative phosphorylation: P:O ratios were determined by the procedure of

SLATER AND HOLTON¹⁰. The reaction mixture contained 0.015 *M* KCl, 0.002 *M* EDTA, 0.02 *M* glucose, 0.03 *M* K phosphate, pH 7.0 or 7.5, 0.025 *M* Tris-acetate buffer⁷, pH 7.0 or 7.5, 0.0001 *M* ATP, 0.005 *M* MgCl₂, 0.05 *M* sucrose, hexokinase (100–150 units⁵), 0.01 *M* glutamate or 0.02 *M* succinate as indicated, and liver mitochondria (3–4 mg protein). The final volume was 1 ml, the reaction temperature 25° and the reaction time 25 min.

P₁-ATP exchange reaction: This reaction^{11,12} was carried out in centrifuge tubes at room temperature (20 ± 2°). The tubes contained (in a volume of 1.45 ml) 0.065 *M* KCl, 0.003–0.004 *M* MgCl₂, 0.001 *M* EDTA, 0.006 *M* ATP, 0.067 *M* Tris-acetate buffer⁷ of the desired pH, and 0.007 *M* phosphate buffer of the same pH containing ³²P. The reaction was started by the addition of 0.05 ml liver mitochondria (in 0.25 *M* sucrose) and after 15 min stopped by the addition of 1.5 ml cold 10% trichloroacetic acid. After centrifugation at 3000 rev./min, the amount of radioactivity in the ATP was determined in a sample of the clear supernatant fluid after separation of the inorganic phosphate from the organic phosphate (ATP) by the method described by NIELSEN AND LEHNINGER¹³. The counting apparatus consisted of a Geiger-Müller tube + automatic scaler (Philips). The samples (0.5 ml) were counted wet in aluminium dishes. After calculation of the total number of counts/min in the ATP, this value was divided by counts/min/μmole inorganic phosphate, yielding the number of μmoles of inorganic phosphate exchanged with the phosphate of ATP. After correction for the breakdown of ATP due to ATPase (amounting to not more than 2%) the exchange was calculated as μmoles/mg protein/h. The amount of mitochondrial protein was generally about 1.0 mg, which gave, under the conditions described, an almost linear exchange with time.

Protein was determined by the biuret method¹⁴ standardized with serum albumin.

RESULTS

Uncoupling activity of mitochrome

Mitochrome, prepared from whole beef liver by the procedure of POLIS AND SHMUKLER², was found to have the same spectral properties as described by these authors (see ELLIOTT *et al.*⁸). Table I shows that we were also able to confirm the uncoupling effect of this preparation on oxidative phosphorylation and the stimulatory effect on the latent ATPase of freshly prepared mitochondria. It also strongly inhibited the P₁-ATP exchange reaction of mitochondria (Table II).

Table II shows that substances which alter the absorption spectrum of mitochrome, namely Na₂S₂O₄, K₃Fe(CN)₆, CO and KCN*, had no effect on the uncoupling activity of mitochrome, which strongly indicated that the haem was not responsible for the uncoupling activity. Moreover, precipitation by heat or by trichloroacetic acid did not destroy the uncoupling activity (Table III). The supernatants obtained by either treatment were inactive. Alcohol, acetone, butanol or isooctane extracts were active. Isooctane was found particularly suitable for extracting the active principle from mitochrome preparations, since a single extraction caused no precipitation of protein. Fig. 1 shows that after extraction of the uncoupling activity into the

* In agreement with other workers, a higher concentration of cyanide (10⁻³ *M*) caused 48% inhibition of the exchange reaction.

TABLE I

EFFECT OF THE MITOCHROME PREPARATION ON OXIDATIVE PHOSPHORYLATION AND LATENT ATPASE OF LIVER MITOCHONDRIA

Oxidative phosphorylation was carried out at pH 7.0 with succinate as substrate. The ATPase reaction was carried out at pH 6.5.

Additions	P:O	ATPase $\mu\text{atoms P/mg protein/h}$
None	1.52	0.3
Mitochrome (0.66 mg protein)	0.01	
Mitochrome (0.33 mg protein)		4.0

TABLE II

EFFECT OF A MITOCHROME PREPARATION AND OF SUBSTANCES REACTING WITH HAEM COMPOUNDS ON THE P_1 -ATP EXCHANGE REACTION OF LIVER MITOCHONDRIA

The pH of the incubation medium was 7.0.

Additions	$\mu\text{atoms P exchanged/mg protein/h}$		Inhibition %
	No mitochrome	Mitochrome (0.16 mg protein)	
None	4.1	0.4	90
$\text{Na}_2\text{S}_2\text{O}_4$ ($10^{-3} M$)	3.5	0.2	94
$\text{K}_3\text{Fe}(\text{CN})_6$ ($10^{-2} M$)	4.3	0.2	95
KCN ($10^{-4} M$)	3.7	0.4	89
CO (100% in gas phase) + $\text{Na}_2\text{S}_2\text{O}_4$ ($10^{-3} M$)	3.5	0.2	94

TABLE III

EFFECT OF VARIOUS FRACTIONS OF A MITOCHROME PREPARATION ON THE P_1 -ATP EXCHANGE REACTION

The pH of the incubation medium was 7.0.

Additions	$\mu\text{atoms P}$ exchanged/mg protein/h
None	4.1
Mitochrome (0.33 mg protein)	0.2
Mitochrome, first precipitated with trichloroacetic acid, then neutralized with KOH and rehomogenized	0.4
Mitochrome, heated at 100° for 10 min (homogenized to make a fine suspension)	0.3
Clear supernatant fluid (neutralized with KOH) of mito- chrome precipitated with trichloroacetic acid	3.4
Clear supernatant fluid of heat-denatured mitochrome	3.5
Ethanol (2.4% final concentration)	3.9
Ethanol extract of mitochrome	0.3
Acetone extract of mitochrome	0.2
Isobutanol extract of mitochrome	0.1
Isooctane extract of mitochrome	0.2

isooctane layer, the spectrum of mitochrome remained unchanged in the water layer. Table IV illustrates that one extraction with isooctane is sufficient nearly completely to remove the uncoupling activity, while the activity of the isooctane extract, like that of the mitochrome preparation itself², is counteracted by the addition of serum albumin.

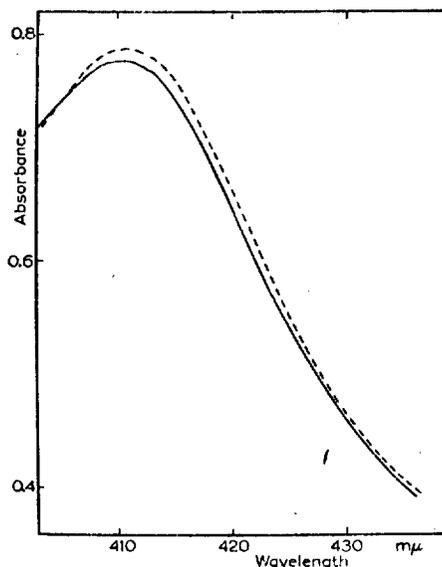


Fig. 1. Effect of extraction with isooctane on the absorption spectrum of mitochrome. ----, unextracted; — extracted. Equal amounts of unextracted and extracted mitochrome inhibited the P_i -ATP exchange reaction by 94% and 34%, respectively.

TABLE IV
EFFECT OF ISOCTANE EXTRACTION ON THE UNCOUPLING ACTIVITY OF A
MITOCHROME PREPARATION

The pH of the incubation medium was 7.0.

Additions	μ atoms P exchanged/mg protein/h
None	6.1
Mitochrome (0.41 mg protein)	0.4
Mitochrome (0.41 mg protein), extracted with iso-octane	4.0
Isooctane extract of mitochrome	0.4
Isooctane extract + 4 mg serum albumin	5.0

Development of uncoupling activity in cytochrome preparations

In the course of the preparation of mitochrome by the method of POLIS AND SHMUKLER², absorption bands of cytochrome *b* were observed with the microspectroscope. However, the bands disappeared when the solution was kept at 4° for a few days. As already described¹, purified preparations of cytochrome ($a + a_3$) and of cytochrome *b*, obtained by the action of crude trypsin in the presence of cholate on a particulate preparation obtained from heart muscle, were transformed to mitochrome on standing for a few days at 4°.

Table V shows that during standing at 4°, there is an increase in the amount of uncoupling material which can be extracted from these cytochrome preparations.

Properties of the uncoupling agent

Tables VI and VII show that the material extractable from mitochondria with isooctane has all the properties of an uncoupling agent. It uncouples oxidative phosphorylation, stimulates ATPase and inhibits the P_i-ATP exchange (Table VI), and also decreases the respiratory-control index (Table VII). The respiratory-control index is the ratio between the rates of respiration in the presence of ADP (maintained by the addition of hexokinase and glucose), and in its absence. Table VII shows the

TABLE V

EXTRACTION OF UNCOUPLING LIPID FROM FRESH AND AGED CYTOCHROME PREPARATIONS

The preparations were extracted with isooctane, and the isooctane extracts added to the reaction medium (pH 7.0) for measurement of the exchange reaction.

Preparation extracted with isooctane	Days at 4°	Exchange (μatoms P _i /mg protein/h)
None	—	6.1
Cytochrome <i>a</i> + <i>a</i> ₃	0	4.7
	7	2.1
	10	1.5
Cytochrome <i>b</i>	0	3.0
	5	0.9

TABLE VI

EFFECT OF ISOCTANE EXTRACT OF A MITOCHROME PREPARATION ON OXIDATIVE PHOSPHORYLATION, ATPASE AND P_i-ATP EXCHANGE OF LIVER MITOCHONDRIA

Reaction	Control	Isooctane extract of mitochondria added*
Oxidative phosphorylation (P:O)**	1.45	0.01
ATPase (μatoms P/mg protein/h)***	0.3	4.0
P _i -ATP exchange (μatoms P/mg protein/h)§	4.1	0.4

* About 17 μg dry wt. (dissolved in 0.04 ml 96% ethanol).

** Succinate as substrate, pH 7.5.

*** pH 6.5.

§ pH 7.0.

TABLE VII

THE EFFECT OF THE ISOCTANE EXTRACT OF MITOCHROME ON RESPIRATORY CONTROL OF LIVER MITOCHONDRIA

Glutamate substrate, pH 7.5.

Isooctane extract	QO ₂		Respiratory-control index
	No hexokinase, Glucose	Hexokinase, Glucose	
0	11.0	52.0	4.7
Approx. 15 μg dry wt.	14.8	48.7	3.3
Approx. 30 μg dry wt.	22.0	43.4	2.0

decrease of this ratio brought about by the addition of the isooctane-extractable material, due partly to inhibition of the respiration in the presence of ADP, and partly to stimulation of the respiration in the absence of ADP.

The first indication of the chemical nature of the uncoupling material was obtained by the experiment shown in Table VIII. To the isooctane-extractable material, dissolved in 4 ml 96% ethanol, was added 1 ml 5 *N* KOH and 25 ml water. The solution was extracted three times with 30 ml petroleum ether, and the combined petroleum ether extract, after washing with water, was evaporated to dryness and taken up in 4 ml 96% ethanol. This solution represented the non-acidic fraction. The alkaline aqueous layer was acidified with HCl to pH 1-2 and extracted with petroleum ether as before. The final extract, dissolved in 4 ml 96% ethanol, represented the acidic fraction. Table VIII shows that practically all the uncoupling activity resided in the acidic fraction. Similar results were obtained when the mitochondrion preparation was extracted with ethanol.

TABLE VIII

DEMONSTRATION THAT UNCOUPLING ACTIVITY OF ISOCTANE AND ETHANOL EXTRACTS OF MITOCHROME RESIDES IN THE ACID FRACTION

The additions given were all dissolved in 96% ethanol. The final concentration of alcohol in the test medium was about 2%. 1.03 mg mitochondrial protein used.

Additions	ATPase (μ atoms P/mg protein/h) at pH						
	5.5	6.0	6.5	7.0	7.5	8.0	8.5
None	0.73	0.41	0.29	0.33	0.63	1.25	1.56
Isooctane extract	2.35	4.31	4.13	3.25	2.28	1.45	1.50
"Non-acid fraction"	1.15	0.88	0.55	0.62	0.93	1.82	1.71
"Acid fraction"	2.30	3.53	3.07	2.07	1.95	1.97	2.00
Ethanol extract	3.78	4.13	3.23	2.37	1.81	1.46	1.64
"Non-acid fraction" of the former	0.92	0.63	0.41	0.55	0.88	1.26	1.50
"Acid fraction" of the ethanol extract	3.89	4.61	3.34	2.41	1.38	1.57	1.96

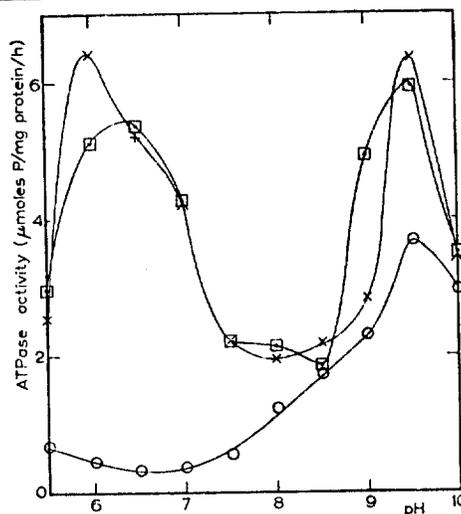


Fig. 2. The effect of the isooctane extract of mitochondrion and of oleic acid on the ATPase activity of liver mitochondria. \circ — \circ , 0.03 ml ethanol; \times — \times , 0.03 ml ethanol containing approx. 30 μ g (dry wt.) isooctane extract of mitochondrion; \square — \square , 0.03 ml ethanol containing approx. 20 μ g oleic acid.

Fig. 2 shows that the pH-activity curves of the ATPase obtained by the addition of the uncoupling agent, and of about the same amount of oleic acid are quite similar. (The actual shape of the pH-activity curve depends upon the concentration of uncoupler; BORST AND LOOS, unpublished.)

Table IX shows an analysis, by gas chromatography, of the acidic fraction of an isooctane extract of mitochondria, after methylation with diazomethane. This analysis was kindly carried out and interpreted by Messrs. R. K. BEERTHUIS AND J. H. RECOURT of the Unilever Research Laboratory at Vlaardingen. It is noteworthy that 61.5% of the acids are unsaturated, a value in close agreement with the 67% found by BALL and his colleagues¹⁵ for the fatty acids present in the lipids of a respiratory-chain preparation isolated from beef-heart muscle. The isooctane extract of mitochondria contains sufficient long-chain fatty acids to account for its uncoupling activity. Quantitative comparisons of the uncoupling activity of various long-chain fatty acids have been carried out by P. BORST AND J. A. LOOS and will be reported elsewhere.

TABLE IX
COMPOSITION OF ACID FRACTION OF ISOOCTANE EXTRACT OF MITOCHROME

<i>Acid</i>	<i>Content</i> % (w:w) of total acid	
<i>Saturated</i>		
C ₁₂	1	
C ₁₄	3.5	
C ₁₅	trace	
C ₁₅ , branched	trace	
C ₁₆	24	
C ₁₇	trace	
C ₁₇ , branched	trace	
C ₁₈	10	
	38.5	38.5
<i>Unsaturated</i>		
C ₁₄	0.5	
C ₁₆ , one double bond	4	
C ₁₈ , one double bond	23	
C ₁₈ , two double bonds	10.5	
C ₁₈ , three double bonds	2.5	
C ₂₀ , highly unsaturated (4 components)	14.5	
C ₂₂ , highly unsaturated (2 components)	6.5	
	61.5	61.5
		100.0

DISCUSSION

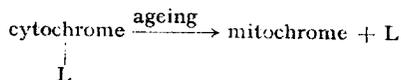
The experiments reported in this paper make it clear that the uncoupling activity of mitochondria preparations resides in an uncoupling agent which can be extracted by organic solvents, rather than in the haem compound mitochondria. This uncoupling

material binds firmly to albumin, which counteracts the uncoupling by both mitochromes and iso-octane extracts. It is interesting that POLIS AND SHMUKLER² found that serum albumin bound the haemoprotein mitochrome so firmly that the two could not be separated electrophoretically. It seems likely that this binding between the two proteins occurs through the lipid uncoupling agent.

The experiment described in Table VIII establishes that the uncoupling lipid material is acidic in nature. Gas chromatography showed that the acidic fraction consisted of a mixture of saturated and unsaturated long-chain fatty acids, oleic acid* (23%), linoleic acid* (10.5%), linolenic acid* (2.5%) and highly unsaturated long-chain acids (21%) comprising 57% of the weight. In studies which will be published elsewhere P. BORST AND J. A. LOOS have shown that oleic acid and linoleic acid are much more highly active as uncoupling agents than the corresponding saturated fatty acids, and, moreover, that there are qualitative differences in the form of the pH-activity curve obtained with oleic and stearic acids, that of oleic acid resembling the curve obtained with the lipid extracted from mitochrome (see Fig. 2). It seems very likely, therefore, that the uncoupling activity of mitochrome can be ascribed to the oleic acid, linoleic acid, linolenic acid and higher unsaturated fatty acids which it contains. It is known that serum albumin firmly binds long-chain saturated and unsaturated fatty acids, particularly oleic acid¹⁶. DAVIS AND DUBOS¹⁷ showed that serum albumin protects erythrocytes against haemolysis by oleic acid.

These findings recall those of PRESSMAN AND LARDY¹⁸ who isolated from microsomes a heat-stable, acetone-soluble fraction which stimulated the respiration of liver mitochondria in a medium deficient in phosphate acceptor, and stimulated the ATPase. The active substances were later identified as a mixture of long-chain fatty acids^{19,20}. PRESSMAN AND LARDY¹⁸ speculated on the possible regulatory role of the microsomal fatty acids on mitochondrial respiration, a possibility which would appear to be strengthened now that we have found similar acids in mitochondrial preparations. This point is further discussed elsewhere²¹.

It seems likely that the uncoupling fatty acids are liberated by hydrolysis of a fatty acid ester present in the mitochondria, and in the cytochrome preparations which are probably derived from mitochondrial membranes. J. A. Loos (unpublished) has found that esterification of the uncoupling fatty acids with diazomethane brought about a loss of uncoupling activity. The mechanism of the liberation of the uncoupling fatty acids is not yet established. The fact that the transformation of isolated cytochrome preparations to mitochrome occurs simultaneously with the liberation of the uncoupling fatty acids could be explained in three ways: (a) the liberation of fatty acids from lipids, possibly an enzymic reaction, occurs independently of the transformation of cytochrome to mitochrome; (b) the following reaction takes place:



where L is written for the unsaturated fatty acid; (c) fatty acids liberated from esters during ageing cause the transformation of cytochrome to mitochrome. Further work is required to enable a choice between these three possibilities.

* The positions of the double bonds in the unsaturated C₁₈ acids shown in Table IX were not determined. Therefore, a positive identification of these acids as oleic, linoleic and linolenic acids was not made.

Recent experiments²² have provided evidence that the release of uncoupling fatty acids in mitochondrial preparations is an enzymic process. The uncoupling can be demonstrated by direct extraction with isooctane of a liver mitochondrial preparation after incubation with 0.001 M CaCl₂ for 90 min at 35°. The release was inhibited by 0.002 M EDTA, 0.002 M ATP and 5·10⁻⁴ M diisopropylfluorophosphate, and prevented by previous boiling of the mitochondrial preparations, or by addition of trichloroacetic acid. Further studies on the nature of the enzyme involved are in progress.

The mechanism of the uncoupling activity of unsaturated fatty acids is not yet clear. It is unlikely to be due to disruption of the mitochondrial structure brought about by the surface activity of the unsaturated fatty acids since the uncoupling is reversible (P. BORST, unpublished).

It appears not unlikely that the stimulation of oxidative phosphorylation brought about by the addition of albumin to suspensions of mitochondria isolated from insect thoracic muscle²³⁻²⁵ or from *Galleria* larvae²⁶ is also due to binding of uncoupling unsaturated fatty acids. This possibility is currently under examination in this laboratory.

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Effects of Dietary Free C₁₈ 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 0.152 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 this heated to

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 chlorine 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 and as the triglycerides.

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	
B	162	197	35	>0.05	>0.05
C	190	313	123	0.02	
D	170	234	64	0.02	0.02
E	197	226	29	—	
F	182	213	31	>0.05	>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.

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Oleic Acid Toxicity and Fat Embolism.*

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In view of the paucity of data on the toxicity of fatty acids, experiments with oleic acid were performed. In control tests, olive oil was injected intravenously, and observations were made pertaining to fat embolism.

The hemolytic effects of fatty acids are a well known phenomenon. This report deals with toxic manifestations observed in addition to those of hemolysis. The intravenous injection of fat emulsion was studied by a number of investigators such as Murlin and Riche;¹ Kochne and Mundel;² Gordon and Levine;³ Clark and Brunschwig;⁴ Dunham and Brunschwig;⁵ McKibbin *et al.*;⁶ Shafiroff and Frank;⁷ LeVeen;⁸ and reportedly by Russian workers who used human fat.

Johnson *et al.*⁹⁻¹¹ have demonstrated in-

creased erythrocyte fragility after the ingestion of large fat meals, due to fatty acids and soaps which enter the blood with the lymph of the thoracic duct.

Dubois *et al.*¹² and Davis¹³ demonstrated that serum albumin has considerable affinity for oleic acid, thereby inhibiting the bacteriostatic effects and toxicity of the latter.

For assays of toxicity, 36 normal, starved, unanesthetized, mongrel dogs were used. A few assays were performed on dogs anesthetized with pentobarbital sodium, in which carotid blood pressures were recorded.

The substances used were oleic acid (95% oleic acid and 5% other long chain fatty acids), ethyl, oleate, and sodium oleate: the latter 2 were prepared from the oleic acid. All substances were administered intravenously. The oleic acid and the ethyl oleate and valuable advice were obtained by the kindness of Dr. A. W. Ralston of the Armour Laboratories.

Results. Doses of 0.25 cc of oleic acid had little effect (6 dogs), while doses of 0.5 to 5 cc were toxic (19 dogs). The outstanding

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symptoms were: drop in blood pressure, urination and defecation, and rapid, forced, deep respirations, coughing, and development of massive pulmonary edema with large amounts of bloody, frothy exudate from the mouth and nose, followed by collapse and death. Life after injections of 5 cc lasted from 5 minutes to 2 hours, with an average of 40 minutes.

Hemolysis occurred, varying in degree from 75 mg % of free hemoglobin to almost complete hemolysis (13.54 gm per 100 ml), depending on the dose of oleic acid given. In animals with low degrees of hemolysis, the hematocrit was elevated. Blood pH usually dropped to the acid side, from 7.46 to 6.37; the lowest value of 6.37 was obtained in a moribund animal, that died within a few minutes. If an animal survived for 1 hour or more, blood pH returned to normal or to alkaline values; the highest alkaline pH observed was 8.2. Serum calcium dropped to 6-7 mg % in most animals, and in 2 dogs to as low as 2 and 0.6 mg %; the latter values appeared in moribund animals that showed the highest degrees of hemolysis.

Autopsy showed the following: the gastrointestinal tract was markedly contracted, especially the pyloric antrum of the stomach; however, the cardia and fundus of the stomach were dilated. The urinary bladder was contracted maximally. The most significant changes were found in the chest. The thoracic cavities contained much bloody fluid. The lungs were edematous and hemorrhagic throughout, and on section bloody fluid oozed from the parenchyma and from the bronchi. The amount of hemorrhage and edema far exceeded any condition which might be ascribed to fat embolism.

Microscopically, in formalin fixed frozen sections, stained with scarlet red and hematoxylin eosin, red masses in the pulmonary vessels varying in size from specks to globules, massive edema of the lungs, and intestinal hemorrhages were observed. Heart, liver, kidneys, and adrenals, were found to contain fat staining material, however not to a strikingly larger extent than tissues from normal controls; that is lipids in the hepatic cells,

in the bile duct cells and in the heart did not appear to be increased or altered by the injection of oleic acid.

Three dogs were injected repeatedly with sublethal doses of oleic acid. They presented the symptoms of increased and forced respiration as described above, but recovered within a day. They seemed to acquire a tolerance to oleic acid which manifested itself by decreasing hemolysis and, after 12 injections, practically no hemolysis was observed. The livers in these chronic dogs were pale yellow and friable, and showed microscopic evidence of fatty metamorphosis.

In order to analyze whether the toxic effect of oleic acid was due to the free carboxyl radical, ethyl oleate was injected in doses of 0.5 to 5.0 cc, (3 dogs). Ethyl oleate was toxic, showing the same clinical symptoms as oleic acid but no edema or hemorrhage of the lungs and no hemolysis, and it was never lethal. Animals sacrificed 1-2 hours after injection did not show the pulmonary changes seen with oleic acid, either grossly or microscopically.

Oleic acid forms soaps in an alkaline medium. In order to analyze the possibility that the toxic effects of oleic acid were due to this, sodium oleate (2 g) was injected (3 dogs). The animals died within a few minutes, showing nearly complete hemolysis.

Comparable amounts of pure olive oil (5 cc) injected intravenously gave no untoward reactions nor hemolysis (2 dogs). Microscopic sections revealed large amounts of fat-staining material in the capillaries of the lungs, much more than with any of the other materials studied, namely oleic acid, ethyl oleate and sodium oleate; yet olive oil was the least toxic of all substances tested. The dogs acted normally and did not seem to be affected in the least. The massiveness of fat embolism as seen microscopically after olive oil injection and the lack of symptoms do not seem to fit too well into the clinical picture of fat embolism.

Conclusions and summary. If, according to the findings of Davis and Dubois, albumin has the power of binding oleic acid in the ratio of 9 to 1, then the amounts of oleic acid in-

jected exceeded the protective range in the dogs used in our experiments.

The mechanism of the toxic action of oleic acid is problematical. Small doses of sodium oleate produced a degree of hemolysis never seen with comparable doses of oleic acid.

While changes in blood serum calcium appeared to be correlated with the degree of hemolysis, the low values of serum calcium may be explained by formation of calcium soaps. Changes of blood pH appeared to be

associated with respiratory conditions and with acid and toxic effects of the oleic acid.

Oleic acid does not seem to produce massive edema of the lungs by embolism, but by toxic effects on the capillaries of the lungs. Caution must be used in the preparation of fat emulsions for intravenous alimentation, in order to exclude fatty acids and soaps. Fat embolism *per se* may not be so dangerous unless fatty acids are released in or into the circulation.

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

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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 re-esterification into chylomicron triglycerides. This

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 $2\text{-}^3\text{H}$ -glycerol-monostearate- $1\text{-}^{14}\text{C}$ in which the stearic acid was in the 1- or 2-position and $2\text{-}^3\text{H}$ -glycerol-1,3-dioleate-2-oleate- $1\text{-}^{14}\text{C}$. The monostearin was synthesized from stearic acid- $1\text{-}^{14}\text{C}$ and glycerol- $2\text{-}^3\text{H}$ 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 $30\ \mu\text{c}/\text{mg}$ for ^3H and $15\ \mu\text{c}/\text{mg}$ 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 ^3H 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 alkalization, which appeared to enhance the isomerization so that assay of the final product instilled showed 20% 2-monostearin and 80% 1-monostearin.

The labeled triolein was prepared from oleic acid- $1\text{-}^{14}\text{C}$ and glycerol- $2\text{-}^3\text{H}$, as previously described (7). For these studies a mixture of $2\text{-}^3\text{H}$ -glycerol trioleate and glycerol-1, 3-dioleate-2-oleate- $1\text{-}^{14}\text{C}$ in the ratio of approximately 10:1 provided a specific activity of $1.6\ \mu\text{c}/\text{mg}$ for ^3H and $0.16\ \mu\text{c}/\text{mg}$ 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 120 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, and 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 streptomycin 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 ethyl-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 introduced 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 2 and 6 hr samples.

¹ Emulphor EL 620, polyoxyethylene tricinoleate, 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 intubation was unsuccessful in this patient. 1 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 nog was 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, neoplasm, 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 BioSonic Model H 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 μ c of oleic acid- 14 C (specific activity 24.6 mc/mm) 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 2 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 1 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- 14 C (40 μ c) obtained from Nuclear-Chicago was mixed with 10 ml of warm water. A micellar solution was made as

follows. Na taurocholate 0.484 g and Na glycocholate 1.288 g (Nutritional Biochemicals Corp., Cleveland, Ohio) were dissolved in phosphate buffer (0.077 M NaCl; 0.005 M Na₂HPO₄). Monoolein 0.50 g, heptadecanoic acid 1.025 g, palmitic acid 0.96 g, and unlabeled glycerol 0.158 g were dissolved in ethyl alcohol:ethyl ether mixture 3:5 (v/v). The buffered bile salts were added to the ether-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 non-radioactive 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% H₂SO₄) 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-diphenyl-oxazole, 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 20,000 μ at 15°C either in a No. 40 rotor or in a No. 20 rotor. The creamy top layer was suspended in buffered phosphate-EDTA solutions and respun as previously described by Kayden, Karinen, and Dument (11). Lymph and chylomicron fractions were separated by thin layer chromatography on Silica Gel G (Binkmann) 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% ^3H 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 $^3\text{H}/^{14}\text{C}$ to meal isotopes $^3\text{H}/^{14}\text{C}$ was initially 1.05, then 0.95, and then fell to 0.69 (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% ^3H 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% ^3H and 36% ^{14}C . Despite a large volume of lymph (360 ml), the sample for the first 2 hr after feeding had little radioactivity (< 0.1% ^3H 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 <i>hr</i>	Lymph volume <i>ml</i>	Triglyceride <i>g</i>	Lymph ^{14}C * Total lymph $^{14}\text{C} \times 100$	$^3\text{H}/^{14}\text{C}$ sample† $^3\text{H}/^{14}\text{C}$ meal	% ^{14}C in 2-position of glyceride
Study I (B.B.) 1:2-monostearin 30:70; total recovery ^3H 53% ^{14}C 57%					
0-2	155	0.44	29	1.05	70
2-4	170	0.90	45	0.95	66
4-6	195	4.64	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 ^3H 35% ^{14}C 49%					
0-2	85	0.26	31	0.98	20
2-4	145	0.73	31	1.00	18
4-8	495	12.4	31	0.70	18
8-12	420	0.61	4	0.42	13
12-16	40	0.09		0.46	16
Study III (W.H.) triolein, labeled fatty acid in 2-position; total recovery ^3H 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	190	5.05	12	0.88	43
15-17	650	5.49		0.77	49
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 ^3H 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	1.26	5	0.94	71
4-6	136	5.06	11	0.86	63
6-8	93	1.72	8	0.88	65
8-10	50	1.17		0.88	61
10-12	15	0.02		0.81	
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- ^3H label; ^{14}C -fatty acid label.

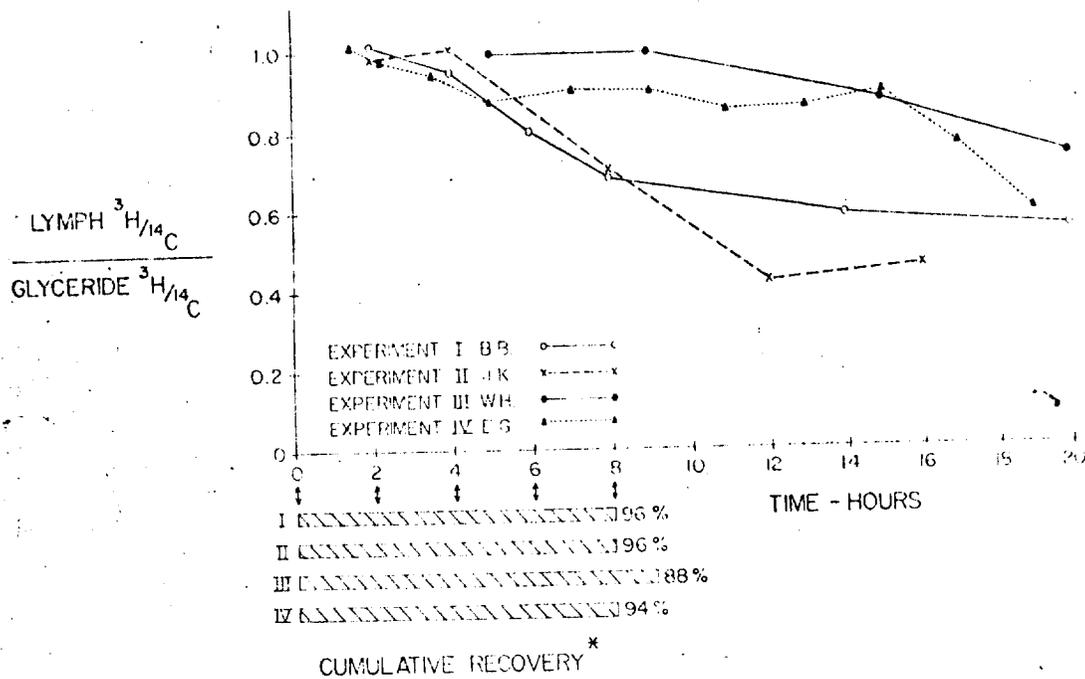


FIG. 1. COMPARISON OF ISOTOPE RATIOS IN HUMAN LYMPH LIPID AND DIETARY TRIGLYCERIDES AFTER GASTRIC OR DUODENAL ADMINISTRATION OF DOUBLY LABELED GLYCERIDES. * *Cumulative recovery* indicates the period during which the indicated per cent of the total labeled fatty acid that was recovered appeared in lymph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

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% ¹⁴C. 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 ¹⁴C 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-¹⁴C was 48%. A tracer amount of labeled free oleic acid along with 20 ml of unlabeled

TABLE II
Composition of thoracic duct lymph after oral administration of labeled (¹⁴C) oleic acid and unlabeled triolein

Time	Lymph volume	Triglyceride	Study V (E.G.); total recovery ¹⁴ C 48%	
			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	52	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	13
4-6	61	1.62	10	18
6-8	66		7	19
8-10	72		4	18
10-12	70		} 12	22
12-16	130			
16-20	110			
20-24	175			

* Figures in this column are the percentage of recovered ¹⁴C.

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	$\frac{\text{Lymph } ^{14}\text{C}^*}{\text{Total lymph } ^{14}\text{C}} \times 100$
hr	ml	
0-0.5	20	0
0.5-1.0	45	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 ¹⁴C.

labeled 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-¹⁴C. 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 ¹⁴C for fatty acids. Mattson and Volpenheim (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 ^3H (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 triolein, supports the concept that greater hydrolysis and reesterification 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 ^3H (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 (16) 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. CUSTOT¹ for a survey of studies published up to 1959. Further important findings were published later²⁻⁷. 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 biological 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 constitutes 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, practically 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 S_j 1 to S_j 4 in charges of 3 kg each; for oils S_j 5 to S_j 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. KAUFMANN¹⁰, peroxide number according to C.H. LEA¹¹, epoxy number according to L. KRULL¹², hydroxy number according to the DGF-unit method¹³, and acid number according to the DGF-unit method¹³.

Feeding test 1 involved the investigation of growth, feed intake, protein efficiency and water intake for the oils S_j K as control, and for the blown oils S_j 1 to S_j 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 23 ± 0°C, and the relative 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 μ thiamine, 100 riboflavine, 1 mg niacine, 100 calciumpantothenate, 50 μ 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
Blaszeiten Std.	—	5	10	15	20	—	2.07	3.0	4.3	7.6
Luftmenge m ³ /kg	—	0.063	0.192	0.104	0.190	—	0.7	1.1	1.7	5.0
Jodzahl	125	121	115	109	109	130	110	96	75	70
Peroxydzahl	3.2	2.0	2.4	1.3	3.7	13.3	14.2	12.1	7.6	7.4
Säurezahl	0.23	0.41	0.55	0.58	0.80	0.0	1.6	2.4	2.8	3.7
Epoxydzahl	0.1	1.30	1.90	1.44	2.60	0.27	6.3	8.1	8.1	8.4
Hydroxylzahl	3.2	2.8	4.4	4.7	8.6	0.0	20.7	27.6	33.0	38.5
Verseifungszahl	192	193	196	193	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 rat 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 resorption.

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. 1.00 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 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 $22^{\circ} \pm 2^{\circ}\text{C}$. 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 OIO 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 $22^{\circ}\pm 2^{\circ}\text{C}$. 14 hours before the start of the test, feed was withheld. Under ether narcosis the abdominal cavity was opened, the intestinal lymph duct is located and freed after careful preparation--as described by J.L. BOLLMANN and collaborators¹⁴-- and a fine PVC-catheter 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 hour 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 (O10) 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 motor system of the stomach-intestine tract.

40 mice weighing 20 g each were used for the tests. Before the tests the animals received LATZ dry feed and water ad libitum. 48 hours before the start of the test the dry feed was withheld and the animals received a 5% glucose-solution and tap water ad libitum. Then untreated and blown soybean oil (O10) was applied to the animals through the esophagus tube in quantities of 0.25 and 0.50 ml per 100 g of body weight, whereby a group of 10 animals was used for each dosage. 2 hours after the application of the oil, 5 ml per 100 g of body weight of an ink solution was administered per os to the animals; the solution was made of 1 part pearl ink and 4 parts water. 40 minutes after the application of the ink the animals were killed and the intestine as well as the stomach removed. The ink conveyance was then verified in the intestine, starting 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 choleresse -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 adequate 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 subsequently unilaterally sealed by melting and centrifuged for 10 minutes at approximately 3000 UpM (revolutions per minute). The serum obtained thereby was transferred into a 20 mm³-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 EPENDORF photometer at a wave length of 578 m μ , 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:

$$R \% = \frac{E_{20}}{E_1} \cdot 100$$

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 KNOLL) 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.L. GREEN and collaborators¹⁶⁾.
- c) Rat liver mitochondrion, prepared in 0.25 ml cane sugar solution according to W.C. SCHNEIDER¹⁷⁾.

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; μ 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, 5 to 6 mg N per mixture; 50 μ mol substratum; 0.013 m phosphate buffer pH 7.4; 0.001 m ATP; 0.0012 m MgSO₄; 0.03 μ 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; 0.013 m KH_2PO_4 ; 0.029 m ATP and 0.015 μ mol 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	43	44	42	40	35
in 4 "	103	98	99	96	88
in 5 "	127	127	126	118	110
Futtermittelverzehr in g					
in 2 Wochen	107	117	107	112	98
in 4 "	248	265	248	260	234
in 5 "	326	350	326	340	309
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 STUDENT¹⁸.

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 statistical evaluation of the tests with slightly blown soybean oils S_j 1 to S_j 4 (Table 2) are significant ($p = 0.001$) only for the weight increase difference between the control group and Group S_j 4. No detrimental effect resulting from the feeding of the less strongly blown oils S_j 1 to S_j 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 S_j 5 to S_j 8 proved to be toxic. As shown on Table 3, a considerable growth inhibition resulted in Group S_j 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 S_j 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 S_j 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 S_j 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 S_j 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 toxicity 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	48
Zahl der Todesfälle	0	0	3	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	0.68
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 "	131	56	41	52	51
in 8 "	150	66	44 *	56	42
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 *	431	459
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.

Growth and protein efficiency during feeding of the blown soybean oil (k)

	1 20% OI in Futter			1 5% OI in Futter			1 2.5% OI in Futter		
	2 Kontrolle	3 gebla-senes Sojaöl	4 geblasenes Sojaöl + Toco-pherol (2 mg/Woche)	2 Kontrolle	3 gebla-senes Sojaöl	6 geblasenes Sojaöl + Toco-pherol (2 mg/Woche)	2 Kontrolle	3 gebla-senes Sojaöl	4 geblasenes Sojaöl + Toco-pherol (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	96 ± 2.4	106 ± 2.8	128 ± 5.3	111 ± 3.9	110 ± 5.1
in 8 " "	125 ± 3.3	—	92 ± 4.7	164 ± 3.6	127 ± 3.8	136 ± 8.1	151 ± 6.4	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	2.03	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	—	1.63	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	3.29	2.37	245	3.71	2.74	216	3.43	2.35	242	3.61	2.22
in 4 "	557	3.27	2.00	557	5.30	2.43	536	5.83	2.60	457	5.32	2.33	486	5.02	2.29
in 6 "	923	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	5.13	1.95	1211	8.58	2.32	—	—	—	1020	9.11	2.29	1066	10.10	2.29
Weibchen 5															
in 2 Wochen 6	233	2.91	1.94	235	3.67	2.34	228	4.05	2.30	233	3.70	2.53	256	4.13	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	8.51	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 of 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 nevertheless 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 determined 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	3 4	3 6	3 8
				Wochen	Wochen	Wochen	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 cm² 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	2	3	4	5
	Magen	Dünndarm	Dickdarm	Magen + Darm	resorbierte Ölmenge in % der Aufnahme
6 3 Std. nach Gabe 7 von unbehandeltem Sojaöl	23.5 ± 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 ± 1.8	6.3 ± 1.8	26.3 ± 2.3	79.7 ± 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

// Mittelwerte von je 10 Tieren; Öl 010

- | | |
|--|----------------------------------|
| 1) Stomach | 6) . . . hours after application |
| 2) Small intestine | 7) of untreated soybean oil |
| 3) Colon | 8) . . of blown soybean oil |
| 4) Stomach + intestine | 9) . . of untreated soybean oil |
| 5) Reabsorbed oil volume
in % of the supply | 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.

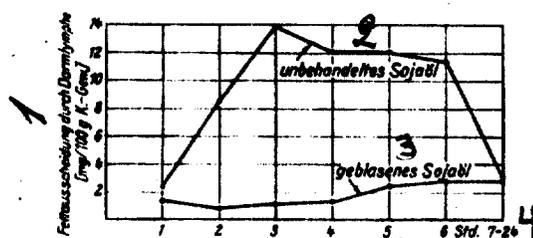


Illustration 1. Fat volume of rats secreted with the lymph after the oral administration of untreated and blown (010) soybean oil (0.25 ml/100 g body weight). (Average values from 6 tests in each case).

- 1) Fat secretion through the intestinal lymph (mg/100 g of body weight)
- 2) untreated soybean oil.
- 3) blown soybean oil;
- 4) 6 hours 7-24

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 intensifies 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
Choleresse (bile) tests with rats. (Oil 010)

Behandlung	Galle-Ausscheidung in % der Leerperiode				
	1.	2.	3. ($\bar{x} \pm s_{\bar{x}}$)	4.	5. Std. nach Ölgabe
unbehandeltes Sojaöl 0.2 ml/100 g intraduodenal (n = 5) ⁴	- 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) 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 hrs 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 ($p = > 0.001$ for the 1st hour; $p = 0.02$ for the 2nd, 3rd and 4th hours; and $p = > .0.001$ 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 choleric effect could depend on the verified prolonged retention of the blown oil in the stomach and intestine.

Secereton 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.

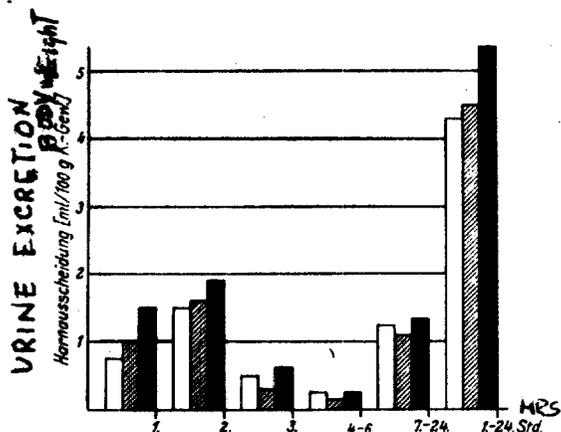


Illustration 2. Urine excretion of rats (average values of 10 animals) after the oral application of 5 ml H₂O/100 g body weight (empty columus) and simultaneous i.p. injection of untreated (shaded columns) and blown (black columns) soybean oil. Oil dosage: 0.5 ml/100 g body weight/ oil 010.

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 Behand- lung	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 Kon- trolle	4.27 ± 0.37 ml	0.241 ± 0.031 mÄq	0.193 ± 0.032 mÄq
7 un- behandeltes Sojaöl 0.5 ml/ 100 g i.p.	4.50 ± 0.14 ml	0.152 ± 0.017 mÄq	0.130 ± 0.015 mÄq
8 geblasenes Sojaöl 0.5 ml/ 100 g i.p.	5.35 ± 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 excretion 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.

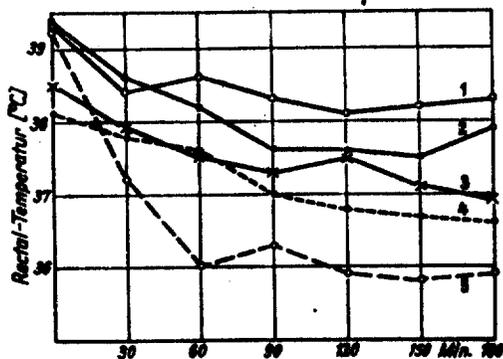


Abb. 3. Rectal-Temperatur von Mäusen nach oraler Gabe von 0.5 ml Sojaöl pro 100 g K.-Gew.

- 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, auto-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 excretion 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 reabsorption 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 thermometer (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 S_j Kw 1; group 2 received the slightly blown soybean oil S_j 1 which proved to be non-toxic during the feeding test; group 3 received the oil S_j 5 which causes considerable growth inhibition but no deaths; groups 4 and 5 received the strongly toxic oils S_j 7 and S_j 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 S_j 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 excluded 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 arterial wall, or whether permeability is indirectly caused by the release of active substances such as histamine, serotonin or similar mediums.

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 it.

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.

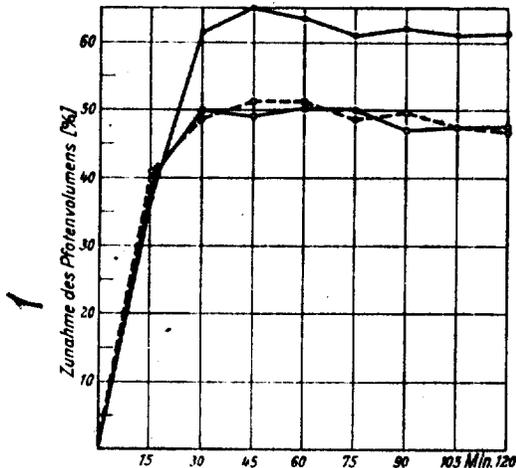


Abb. 4. Intensität und zeitlicher Verlauf des Dextran-Pfoten-ödems von Ratten

- 2 0...○ ohne Vorbehandlung (Kontrolle)
- 3 0—○ nach oraler Gabe von 0.5 ml unbeh. Erdnußöl/100 K.-Gew.
- 4 0—● nach oraler Gabe von 0.5 ml oxyd. Erdnußöl/100 K.-Gew.
- 5 0—○ Ulagabe jeweils 90 Min. vor Dextran-Injektion;
- 6 Mittelwertskurven aus jeweils 6 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 soybean oil 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 dye-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	
		\bar{x}	$\pm \bar{s}$
4 Sj KW ₁ 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. GERLICH¹⁰ 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 proportionately 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.

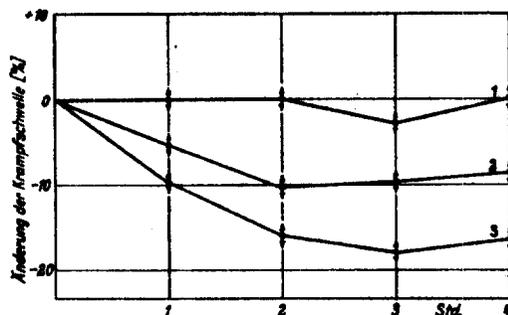


Abb. 5. Änderung der Krampfschwelle bei Ratten nach oraler Gabe von unbehandeltem und geblasenem Erdnußöl (5 ml/kg K.-Gew.)

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 (%)
- 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 evaluated 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.

		<i>Rattenaktivität nach oraler Gabe (0.5 ml/100 g) von unbehandeltem und geblasenem Erdnußöl</i>		
1 Erdnußöl		2 Kontakte/5 Std.		
		\bar{x}	$s\bar{x}$	P
3	unbehandelt	2131	± 269	—
4	1 Std. geblasen	2435	± 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	" "	3348	± 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, monoentials and dienals are contained in said fraction, i.e. substances with very strong reactivity²⁰⁻²⁴. 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 enzyme-preparations.

TABLE 13

Inhibition of cell respiration by the fraction of volatile substances which form during the blowing of soybean oil.

1 Substrat	2 Enzym- präparat (Rattenleber)	3 Zahl der Versuche	Beobachteter % Hemmung	4 % Akti- vierung
Citrat	Cyclophorase	1	23	—
α-Ketoglutarat	Cyclophorase	4	50, 86, 100, 100	—
Succinat	Cyclophorase	1	46	—
	Mitochondrien	1	83	—
Fumarat	Cyclophorase	1	95	—
Malat	Cyclophorase	4	14, 37, 64, 97	—
	Mitochondrien	2	21, 35	—
L-Glutamat	Cyclophorase	2	100, 100	—
	Mitochondrien	2	100, 100	—
Capronat	Cyclophorase	1	100	—
5 endogene Substrate (Leeratmung)				
	Cyclophorase	7	43, 44, 45, 46,	—
	Enzympräparat		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.

25-27

E. SCHUAUENSTEIN 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 earlier 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 S_j 5 to S_j 8 in the course of the feeding tests. The groups S_j 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 a 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 Körper- gewicht in g	3 Darm- trakt	4 % des Körpergewichtes				
			5 Herz	6 Leber	7 Milz	8 Niere	9 Testes
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	1.03	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	1.13	—

14 Angaben in % des Körpergewichtes; Mittelwerte

- | | |
|---------------------|---|
| 1) Group | 10) Males |
| 2) Body weight in g | 11) Controls |
| 3) Intestinal tract | 12) Females |
| 4) % of body weight | 13) Controls |
| 5) Heart | 14) Data in % of body weight; average values. |
| 6) Liver | |
| 7) Spleen | |
| 8) Kidney | |
| 9) Testicles | |

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 thus be transferred into the lumen of the small urethra. Most of it, however, remains at the original site. These masses can reach a diameter 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 (Spermiën) 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. KANNAS³⁰. 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 surrenal 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 surrenal 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. HINTZSCHE³¹. 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.

	<u>1</u> Kontrolle Sj KW	<u>2</u> Gruppe Sj 8
<u>3</u> Zahl der Zellkerne in 0.225 mm^2	91.3	80.3
<u>4</u> Durchschnittliches Kern- volumen in μ^3	131	132

³⁰ Acta endocrinol. 11, 49 [1952].

³¹ Experientia (Basel) 1, 4 [1945].

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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, E. DEGRWITZ and K. LANG³² 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 can, however, weaken said effect in some cases. Furthermore, it was found that the feeding of blown oils causes a certain deficiency of essential fatty acids in the organism. It should therefore be discussed whether part of the symptoms could be attributed to the essential fatty acid deficiency of the organism and also to an absolute or relative tocopherol deficiency.

Various laboratories, including our own, among others³², recently demonstrated that the body fat of humans and animals contains substances which are colored again by decolorized dichlorophenol-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 oils³². 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 encephalomalacie³⁷⁻³⁸.

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. Blowing nevertheless reduced the dien-acid content of the soybean oil used as feed to 16%. But when the supplied feed volume is taken into consideration, a daily dose of 300 mg linolenic acid still results per rat, i.e. roundly the 15-fold of the amount needed.

Despite the high supply volume the animals were unable to maintain the linolenic acid reserve of their organism. During this test the initial trien-acid increase in the organic fat did even indicate an incipient deficiency condition concerning the essential fatty acids; however, during the intake of a 15-fold volume of the normal daily need said condition could not have been due to external causes; 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 fat³² 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 2.5% 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 0.45 to 0.90 g per kg of body weight. Referred to a human weighing 70 kg, the daily blown fat volume would amount to 31.5 to 63 g. The toxicity at 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 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 toxicity 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 cell 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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Pathology

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CYTOTOXIC EFFECT OF SODIUM OLEATE. R. Kinoshita, H. Miyaji,*
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One intraperitoneal injection of 10 mg sodium oleate in 1 ml water caused hitherto undescribed changes in the liver of the rat. Within a few days, the liver became plump with rounded edges. Cytoplasm of the liver cells contained unstained patches of irregular shape. Electron microscopy of these areas demonstrated the lack of ribosomes and alteration of the endoplasmic reticulum, which, instead of cristae, formed a network of narrow canals or became fuzzy and filamentous. The cristae and membranes of mitochondria were indistinct. Eventually, the liver cells regained their usual structures. The liver remained plump in form, unaccompanied by cirrhotic changes. Marked dilatation of the central veins and hyperplasia of the liver cells was evident. Females were more sensitive to the compound than males. Fatty acids with 8 or more carbons were effective; those with 12 and 14 carbons were most active. Saturation and hydroxylation altered the results very little.
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The Digestibility of Fats¹

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NOTWITHSTANDING the fact that fats and oils are an important source of energy in the diet and are two and one-fourth times as effective for this purpose as either proteins or carbohydrates, their dietetic use has received less attention at the hands of investigators than other food constituents, and is consequently less well understood.

The general assumption has been that when eaten in favorable combinations fats are well assimilated and that they do not vary enough in this respect to affect materially the amount of energy which the body receives from them. Experimental data are limited also with respect to the relation of melting point to thoroughness of digestion, particularly in the case of fats with a high melting point. This is a matter of particular interest, since increased demand for culinary and table fats tends to bring into the market kinds which hitherto have been used little, if at all.

PREPARATION OF TEST MEALS

Since it was rarely possible to find a subject who could eat fats and oils in quantity in their natural state, a method of preparing them was sought which did not submit the fats to high temperature for any considerable time, was applicable to the different kinds of fat, gave a reasonably palatable dish, and could be prepared in quantity sufficient for the entire experimental period. A method which proved satisfactory was to incorporate the fats in a sweet cornstarch pudding or blanc-mange. This was prepared by cooking in an ordinary double boiler a mixture of skim milk and sugar to which a considerable quantity of very dark caramel solu-

Because of the relation of fats to agriculture and their great importance in the diet, a long series of experiments on the thoroughness of digestion of animal and vegetable fats has been included in the studies of food and its uses in the home, which were made in the Office of Home Economics. The work has been carried on at intervals for several years and has been reported in bulletins of the U. S. Department of Agriculture and in other publications.²

The studies have included twenty-three animal fats, thirty-four vegetable fats, and six hydrogenated oils. In all cases the goods were bought in the open market or specially prepared either in the Department of Agriculture laboratories or in some other way which assured full knowledge of the process.

tion was added. When this had reached its maximum temperature (nearly 100° C.), a mixture of cornstarch and the fat under consideration was slowly added with constant stirring and the heating was continued until the starch seemed well cooked. The caramel solution gave the pudding a slightly bitter taste, but this could be readily overcome by add-

ing a little vanilla extract when the pudding had cooled. The cornstarch pudding prepared in this way resembles a common household dessert except that it is not noticeably sweet. By this method it is possible to mask to a large extent the texture and flavor of the fats and oils. The good results obtained in this laboratory with this cornstarch mixture as a medium for supplying fat and other special foodstuffs to the diet indicate that it might prove valuable in invalid dietetics, particularly where a patient has an aversion to a large amount of fat or other foodstuff.

The cornstarch mixture was used for all the fats and oils here considered except fish and avocado fat. In these two cases the fat was not separated out. The fish meat, fat and lean together, was freed from bone and made up into fish loaf, such as had been successfully used in previous similar experiments in this laboratory. The fresh avocado pulp was fed simply with a little salt or lemon juice, as is a common custom.

Experience has shown that in such digestion experiments a simple mixed diet is more pleasing to the subject than a single food; consequently, in this work with fats the cornstarch pudding was supplemented with a very simple basal diet consisting of a commercial wheat biscuit or cracker, some fruit, and tea or coffee with a little sugar if the subject desired it.

PROCEDURE

A survey of the literature clearly shows that some variation in results can be expected in digestion experiments with different procedures, the analytical methods followed having some effect, for instance, on the determination of the quantity of fat extracted. There is also some difference of opinion regarding the desirable length of digestion periods and the methods of separating the feces; therefore the results of digestion experiments will vary within limits according to these differences in methods. However, from the extended experience, covering some twenty-five years or more, in which such problems have been studied in connection with the Department of Agriculture's food work, it is fair to conclude that where the same experimental procedure is followed, the results with various food materials are directly comparable.

The method developed and followed in this laboratory is one of several, the value of which is generally recognized. No method yet devised is absolute. Our experience, however, leads us to believe that any of these methods consistently followed throughout a series of experiments will yield re-

¹ Presented before the Division of Food Chemistry at the 64th Meeting of the American Chemical Society, Pittsburgh, Pa., September 4 to 8, 1922.

² "Digestibility of Some Animal Fats," *U. S. Dept. Agr., Bull.* 310. (Beef fat, butter, lard, mutton fat.)

"Digestibility of Some Vegetable Fats," *U. S. Dept. Agr., Bull.* 305. (Cocoa butter, coconut oil, cottonseed oil, olive oil, peanut oil, sesame oil.)

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"Digestibility of Some By-product Oils," *U. S. Dept. Agr., Bull.* 731. (Apricot-kernel oil, cherry-kernel oil, melon-seed oil, peach-kernel oil, pumpkin-seed oil, tomato-seed oil.)

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liable results and that the range of variations between individual tests with the same kinds of material will be similar whatever the method. In order to give comparable results, however, any method must be applied in essentially the same way in every case.

TABLE I—DIGESTIBILITY OF ANIMAL FATS

FAT USED IN TEST	Melting Point ° C.	No. of Tests	DIGESTIBILITY			Corrected for Metabolic Products and Fat of Basal Ration Per cent
			Highest Per cent	Lowest Per cent	Average Per cent	
Butter fat	32	8	95.7	90.9	93.9	97.0
Cream	..	7	95.2	89.3	92.7	96.9
Goat's butter	..	4	94.8	92.4	93.6	98.4
Lard	35	9	95.7	88.2	93.7	97.0
Beef suet (rendered)	45	10	94.4	82.4	88.9	93.0
Beef bricket	..	7	95.9	89.1	92.8	97.4
Hard-palate fat of cattle	34	3	91.7	88.8	90.5	93.7
Ox-marrow fat	..	4	93.3	89.3	91.2	93.5
Ox-tail fat	36.8	2	95.0	89.0	93.0	96.6
Mutton suet (rend.)	50	7	88.2	72.4	80.5	88.0
Kid fat	..	3	95.2	87.7	91.3	95.3
Horse fat	..	3	95.0	92.4	93.6	93.9
Deer fat	49-54	3	75.4	71.5	73.9	81.7
Chicken fat	..	8	95.8	92.0	93.4	96.7
Goose fat	..	7	96.5	91.6	94.2	95.2
Oleo oil	..	8	92.5	87.4	90.4	96.8
Oleostearin	..	3	79.0	66.4	71.8	80.1
Egg-yolk fat	..	6	92.1	90.9	91.5	93.8
Fish fat	Room temp.	3	96.2	94.2	95.4	96.1
Turtle fat	..	4	94.5	91.8	93.3	98.6
Cod-liver oil	Room temp.	4	93.8	88.9	92.6	97.7

In the work under consideration the experimental period covers three days. The feces are marked for separation with charcoal or carmine, or both supplied with the first meal of the experimental period and the first meal after it. The feces for the entire period are dried, thoroughly mixed, and sampled, and analyzed by the standardized methods of the Association of Official Agricultural Chemists. In calculating the results, corrections are made for the small amount of fat introduced in the skim milk used in the blanching and in the basal ration, and also for metabolic products in the feces, the factors used being based on what is believed to be ample experimental evidence. Uniformity is also observed in the choice of subjects. Generally speaking, they

TABLE II—DIGESTIBILITY OF SOME VEGETABLE FATS

FAT USED IN TEST	Melting Point ° C.	No. of Tests	DIGESTIBILITY			Corrected for Metabolic Products and Fat of Basal Ration Per cent
			Highest Per cent	Lowest Per cent	Average Per cent	
Olive oil	18	15	95.6	91.5	94.7	97.8
Cottonseed oil	..	12	94.9	91.6	94.9	97.6
Corn oil	..	7	94.9	88.6	93.5	96.9
Sesame oil	..	5	95.3	92.1	93.8	98.0
Soy-bean oil	..	7	95.8	92.4	93.8	97.5
Sunflower-seed oil	Room temp.	4	95.2	91.9	93.8	96.5
Japanese mustard-seed oil	..	3	96.6	92.6	94.9	95.8
Rapeseed oil	..	4	96.6	93.7	95.7	98.8
Charlock (wild mustard) seed oil	..	4	96.3	93.7	94.6	98.9
Cocoa butter	30	11	95.5	79.1	89.6	94.9
Peanut oil	..	5	97.6	93.7	96.0	98.3
Coconut oil	..	12	96.3	88.1	93.5	97.9
Almond oil	..	4	96.2	86.3	93.1	97.1
Black-walnut oil	..	4	96.7	91.9	94.8	97.5
Brazil-nut oil	..	3	95.8	92.3	94.5	96.3
Butternut oil	..	3	94.7	85.3	88.7	95.4
English-walnut oil	..	3	95.6	92.5	94.4	97.6
Hickory-nut oil	..	4	98.4	96.5	97.2	99.3
Pecan oil	..	4	96.9	90.1	93.9	96.8
Apricot-kernel oil	Room temp.	4	95.4	92.3	94.4	98.4
Cherry-kernel oil	..	4	95.5	93.0	95.2	98.0
Peach-kernel oil	..	3	96.5	87.0	91.6	96.6
Watermelon-seed oil	..	3	94.2	88.5	90.7	94.8
Melon- (cantaloupe) seed oil	..	3	97.7	92.1	94.8	98.2
Pumpkin-seed oil	..	2	95.0	94.9	95.0	98.2
Tomato-seed oil	..	3	91.3	88.1	90.3	95.8
Palm-kernel oil	..	4	97.5	93.9	95.3	98.0
Hempseed oil	..	3	94.7	94.0	94.4	98.5
Poppy-seed oil	..	7	93.4	87.4	91.3	96.3
Coburne oil	..	4	94.7	94.0	94.6	99.1
Cupuaçu fat	Near 28	4	91.0	86.3	88.8	94.1
Avocado fat	..	3	90.8	88.7	90.0	97.9
Java-almond oil	..	2	95.5	89.0	92.2	97.0
Tea-seed oil	Room temp.	1	88.2	91.2

have been laboratory assistants or students of local medical or dental schools who had an intelligent interest in the work, which was a decided help in conducting the experiments. In a general way the subjects had more or less knowledge of the problems studied, but they were not given any definite information regarding the special foods under consideration.

RESULTS

In summing up the results it has seemed desirable to group the fats according to their origin, and the tables which follow show the coefficients of digestibility of animal fats in Table I, of vegetable fats in Table II, and of oils hydrogenated to a definite point in comparison with a blend obtained by mixing a softer fat with one hydrogenated to a high melting point in Table III.

TABLE III—DIGESTIBILITY OF HYDROGENATED FATS

FAT USED IN TEST	Melting Point ° C.	No. of Tests	DIGESTIBILITY			Corrected for Metabolic Products and Fat of Basal Ration Per cent
			Highest Per cent	Lowest Per cent	Average Per cent	
<i>Oils Hydrogenated to Definite Melting Points</i>						
Hydrogenated corn oil	33.0	5	95.0	87.6	91.7	94.7
	42.0	5	98.8	89.4	91.8	95.4
	50.0	5	91.2	78.9	82.2	88.5
Hydrogenated cottonseed oil	35.0	5	96.9	89.7	93.6	96.8
	38.6	1	92.7	..	92.7	95.5
	46.0	3	93.8	91.0	92.7	94.9
Hydrogenated peanut oil	37.0	5	96.9	94.0	95.0	98.1
	39.0	3	95.4	90.2	93.3	95.9
	43.0	5	96.9	90.1	93.5	96.5
	50.0	4	90.3	86.6	88.1	92.0
	52.4	3	81.8	65.8	73.8	79.0
	<i>Hydrogenated Oils Blended to Definite Melting Points</i>					
Blended hydrogenated corn oil	39.0	4	94.9	88.5	92.1	95.2
	49.0	3	92.4	87.0	90.4	93.3
	54.0	3	90.1	83.5	87.8	91.5
Blended hydrogenated cottonseed oil	41.3	2	92.8	91.9	92.4	96.6
	45.8	2	93.8	90.5	92.2	96.4
	47.8	4	94.0	85.4	90.7	94.2
	48.1	2	90.2	89.5	89.8	94.4
Blended hydrogenated peanut oil	50.0	3	85.6	76.3	82.0	87.0
	43.0	3	94.2	91.5	92.7	96.6
	44.2	4	97.0	91.3	94.4	97.4
	51.1	4	91.8	87.2	89.8	92.8

Consideration of the figures shows that ordinarily there are no very great differences in the digestibility of the fats studied. The animal and vegetable fats and the oils liquid at ordinary room temperature, for instance, have very much the same digestibility. It is apparent, however, that fats and oils melting above the body temperature are not quite so thoroughly assimilated, and the observed data seem to warrant the conclusion that the thoroughness of digestion is inversely proportional to the melting point. Experiments with the hydrogenated fats indicate that fats hydrogenated to a definite melting point and those made by mixing a softer fat with one hydrogenated to a much higher melting point do not differ essentially in thoroughness of digestion.

It was very noticeable in this extended series of digestion experiments and in similar series with other foods that the diet did not become monotonous and that the regularity in the diet and all that pertained to it was not irksome or disturbing. The amount of fat eaten per person per day varied within considerable limits, 50 to 60 g. being regarded as a small quantity per day, and 110 or 115 g. as a generous amount. Seldom, if ever, was any physiological disturbance noted when smaller amounts were eaten, nor was any observed in the majority of cases when the larger amounts were taken. However, it seems clear that in some cases, because of the nature of the fats themselves or because of unidentified substances accompanying them, they exercised a more or less laxative effect. For instance, this effect was observed when 140 g. of rendered beef suet were eaten per day, and was not noticeable with butter, lard, or mutton fat used in the same series of tests. In the case of olive oil and most

of the other oils used, as much as 116 to 130 g. were eaten per day with no laxative effect. In the case of cocoa butter, however, a decidedly laxative effect accompanied by slight nausea was noted, indicating that the limit of tolerance of this fat is not so high as that of the other oils. A similar effect was also noted with cupuassu fat, which comes from a tree rather closely related to the *theobroma cocoa*. It is also worth mentioning that a laxative effect was noted when goose fat was eaten in quantity but was not noted with a comparable amount of chicken fat. It seems not unlikely that studies undertaken to explain these observed divergencies would yield very interesting results.

CONCLUSION

From the investigations as a whole it seems fair to conclude that fats are wholesome, useful foodstuffs, which are well assimilated, and that for such reasons they deserve the im-

portant place they have in the diet. The fact that they are often carriers of vitamins is another reason for the esteem in which they are held.

No attempt can be made here to discuss their effect upon palatability of the diet and their important relation to the texture, flavor, and quality of the foods into which they enter, though these and other matters relating to fats are being studied in the Office of Home Economics, where it is also proposed to continue the work on the digestibility of fats when new ones or new processes of manufacture add to the list of material available for such purposes.

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No account of this work carried on in the Office of Home Economics would be complete without noting the fact that much of its success is due to Mr. Arthur D. Holmes and Mr. Harry J. Deuel, who assumed direct charge of the experiments.

An Endogenous Uncoupling and Swelling Agent in Liver Mitochondria and Its Enzymic Formation*

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Physical or chemical disruption of the structure of liver mitochondria usually leads to preparations with considerable respiratory activity, but with little or no activity in coupling phosphorylation to respiration. Under carefully controlled conditions, however, submitochondrial preparations with considerable phosphorylating activity may be obtained (1-5). At least some of the loss of phosphorylating activity which ordinarily accompanies mitochondrial disruption may be caused by uncoupling factors which are released or become active as mitochondrial structure is altered. In a search for such endogenous factors, two distinct activities of this general type were observed (6). The first, R factor, is a protein-containing factor which releases respiration of mitochondria or mitochondrial subfragments from its normal dependence on the presence of a phosphate acceptor system, but which does not uncouple phosphorylation (6). The second agent, here designated as U factor, which is heat-stable in nature and readily extracted from mitochondrial fractions with isooctane and other solvents, has been found to uncouple phosphorylation, release respiration from control by adenosine diphosphate, and cause the swelling of mitochondria. These effects of U factor may be prevented by the presence of bovine serum albumin. This paper is concerned with the assay, general properties, and enzymic formation of U factor in rat liver mitochondria. The possible identity of U factor with the active principles of the mitochondrial uncoupling preparations of Pullman and Racker (7), Polis and Shmukler (8), and Hülsmann *et al.* (9) is also pointed out.

EXPERIMENTAL AND RESULTS

Rat liver mitochondria were isolated from sucrose homogenates as described before (10) and were washed 3 times; these served as starting material for the preparation of U factor and the phosphorylating digitonin fragments (10) used to assay the U factor activity.

Preparation of U Factor—A standard preparation of U factor in isooctane solution was used for many of the experiments described below. It was prepared as follows. The washed mitochondria isolated from 60 gm. of rat liver were suspended in 0.03 M KCl to make a total volume of 60 ml. The suspension was subjected to sonic oscillation for 30 minutes at 4° in the

cavity of a 9 kc. Raytheon magnetostriction oscillator. The sonically disrupted mitochondria were then incubated at 23° for 2 hours. The suspension was brought to pH 3.0 with 5 N HCl and extracted 3 times with equal volumes of isooctane (2,2,4-trimethylpentane, "spectro" grade, Eastman). The emulsions formed were broken by freezing and thawing followed by centrifugation. The clear, colorless isooctane extracts were combined and stored at 0°, with no change in activity for months. This extract, was assayed as described below, contained approximately 2.5 units of U factor per ml.

Assay of U Factor Activity—The ability of U factor to uncouple oxidative phosphorylation was used as a basis for a semi-quantitative assay. Ordinarily, U factor was first extracted from aqueous mitochondrial suspensions or extracts by shaking 3 times with equal volumes of isooctane, following the general procedure described above. Usually the aqueous phase was first brought to pH 3 to 4 with HCl prior to extraction, although essentially complete extraction was also observed at pH 6.5. The combined isooctane extracts were made to a standard volume.

The enzymic assay of U factor present in isooctane extracts was carried out as follows. Small aliquots of the isooctane extract, between 0.1 to 5.0 ml., were placed in 20-ml. beakers of the type used in the Dubnoff shaker. These extracts were then evaporated to dryness by placing them in a large desiccator attached to a water aspirator. The isooctane-extractable material was thus deposited in a thin, invisible layer in the beaker. The components of the enzymic test system were then pipetted directly into the prepared beakers and the medium shaken in the beaker for 15 minutes at 20° before the addition of the enzyme to dissolve or suspend the uncoupling factor.

The enzyme test system contained 0.01 M DL- β -hydroxybutyrate, 0.0024 M ADP, and 0.03 M phosphate buffer, pH 6.5, labeled with P³² ($\sim 2 \times 10^5$ c.p.m.). A water suspension of phosphorylating digitonin fragments from rat liver (10), containing about 200 μ g. total N, was added last to complete the system which had a total volume of 2.0 ml. Incubation was carried out at 20° for 15 to 20 minutes. The reaction was stopped with trichloroacetic acid and the phosphate uptake and acetoacetate formation measured as described before (10); the data were used to calculate the P:2e ratios. The control P:2e ratios, obtained in the absence of added U factor, were between 1.5 and 2.0.

U factor activity was expressed in terms of arbitrary units: 1 unit was defined as the amount required to lower the P:2e ratio in the above test system to 50 per cent of its control value. Ordinarily two or three levels of each sample of U factor were

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TABLE I

Uncoupling of phosphorylation and respiratory release by U factor; action of serum albumin

The test system for oxidative phosphorylation contained 0.01 M DL- β -hydroxybutyrate, 0.0024 M ADP, 0.01 M histidine pH 6.5, 0.03 M phosphate buffer pH 6.5 (labeled with P^{32} , $\sim 1 \times 10^5$ c.p.m.), 0.01 M glucose, 1.0 mg. hexokinase preparation; 0.001 M EDTA, digitonin fragments (215 μ g. N), and U factor equivalents as shown below in total volume of 2.0 ml. Serum albumin was added at 4.0 mg. per vessel as shown. The test system for release of respiration contained only 0.01 M β -hydroxybutyrate, 0.01 M histidine pH 6.5, 0.001 M EDTA, digitonin fragments (215 μ g. N), and U factor as shown to total volume of 2.0 ml. All vessels were incubated 20 minutes at 20°. Aliquots of U factor solution in isooctane (2.2 units per ml.) were evaporated in reaction vessels as shown.

U factor	Serum albumin	Acetoacetate formed		P:2e
		No acceptor	Plus acceptor	
ml.		μ moles	μ moles	
0.0	—	120	324	2.24
0.3	—	178	318	1.86
0.5	—	218	328	0.94
0.7	—	268	334	0.54
1.0	—	290	326	0.14
1.5	—	308	332	0.06
1.5	+	102	338	2.30
6.0	—	94	88	0.02
6.0	+	122	306	2.02

TABLE II

Effect of U factor on ATP exchange reactions and ATPase

The test system for the ATPase and ATP- P_i^{32} exchange reaction contained 0.006 M ATP pH 6.5, 0.0004 M phosphate labeled with P^{32} (1.22×10^5 c.p.m.) and digitonin fragments (208 μ g. N) in a total volume of 2.0 ml. A 1.5-ml. aliquot of U factor in isooctane (3.5 units) was evaporated in the vessel beforehand. The incubation period was 20 minutes at 20°.

The ATP-ADP exchange was measured in a system containing 0.004 M ADP- C^{14} (14,000 c.p.m.), 0.006 M ATP, pH 6.5, and digitonin fragments (55 μ g. N) (aged 72 hours at 0° (11)) in total volume of 0.5 ml.; 1.75 units of U factor were added as shown. The incubation period was 20 minutes at 20°. Serum albumin was added as shown at a level of 2.0 mg. per ml.

Experiment No.	Activity		
	No addition	U factor	U factor + serum albumin
	μ moles	μ moles	μ moles
1. ATP- P_i^{32} exchange, ATP 32 formed.....	112	6	136
2. ATP-ase activity, P_i formed.....	320	540	284
3. ATP-ADP exchange, ATP- C^{14} formed.....	370	370	370

assayed and the unitage arrived at by extrapolation if necessary. The activity of a sample could thus be established to within about 25 per cent.

Action of U Factor on Oxidative Phosphorylation and on Respiratory Control by ADP—The typical data collected in Table I show that preparations of U factor extracted from sonically

disrupted and aged rat liver mitochondria as described previously cause uncoupling of phosphorylation and release of respiration from its dependence on the presence of a phosphate acceptor system, when tested with suspensions of digitonin fragments from rat liver mitochondria. Both the uncoupling and release of respiration are dependent on concentration of U factor; at the higher levels tested uncoupling and release of respiration were essentially complete. Furthermore, it is seen that the release of respiration by U factor resembles that given by dinitrophenol (6) in that the extent of release of respiration in the absence of acceptor is parallel and roughly proportional to the extent of uncoupling when acceptor is present. The action of U factor thus is different from that of R factor; the latter causes respiratory release but does not uncouple phosphorylation significantly (6).

The data in Table I also show that bovine serum albumin completely prevents the respiration-releasing activity and also the uncoupling activity of the highest level of U factor added to the test systems. When great excesses of U factor were added in the absence of serum albumin, substantially greater than required to uncouple phosphorylation completely, the respiration was severely inhibited; a typical experiment is shown in Table I. This effect is also prevented by serum albumin.

Effect of U factor on ATPase and ATP Exchange Reactions—U factor also causes the inhibition of the ATP- P_i^{32} exchange reaction and the stimulation of ATPase activity of the digitonin fragments as shown by the data in Table II. In these respects U factor also resembles the action of dinitrophenol. These effects of U factor are completely prevented by an excess of bovine serum albumin as shown by the data. Great excesses of U factor in the absence of serum albumin caused substantial inhibition of ATPase; its action is therefore biphasic as is its action on respiration.

The data in Table II show, however, that the ATP-ADP exchange reaction as it occurs in aged digitonin fragments (11) is not inhibited by U factor. Under these conditions dinitrophenol likewise has no effect on this exchange (11).

From these findings it appears likely that U factor at the concentration levels tested is an uncoupling agent that has effects very similar to those of dinitrophenol, suggesting that it acts at the same point in the coupling mechanism, presumably near the carrier level (11–13). U factor thus differs in its action from other uncoupling agents such as gramicidin, arsenate, and azide which have characteristically different actions on the ATP exchange reactions and ATPase activity (11, 12).

Mitochondrial Swelling Caused by U Factor—U factor causes a great acceleration of the swelling of rat liver mitochondria (cf. 14–16) suspended in a medium of 0.125 M KCl-0.02 M Tris buffer at pH 7.4, as is shown in Fig. 1. Approximately 0.2 unit of U factor caused swelling of a 50 mg. of tissue equivalent of rat liver mitochondria, equal to the rate produced by 1×10^{-5} M L-thyroxine (14) or 3×10^{-6} M sodium oleate. As seen in Fig. 1, the swelling produced by U factor was completely prevented by the presence of 1.0 mg. per ml. of bovine serum albumin in the test medium.

Effect of Sodium Oleate—Sodium oleate solutions appear to be capable of annulling a set of the effects of U factor with respect to uncoupling, inhibition of the ATP- P_i^{32} exchange, stimulation

¹ The abbreviations used are: EDTA, ethylenediaminetetraacetate; P_i , inorganic orthophosphate; Tris, tris(hydroxymethyl)aminomethane.

of ATPase activity, release of respiration from its dependence on the presence of a phosphate acceptor system and mitochondrial swelling. The uncoupling activity of a 1.0 unit of U factor could be reproduced by approximately $0.03 \mu\text{mole}$ of sodium oleate. Similarly the mitochondrial swelling activity of a 1.0 unit of U factor could be produced by about $0.03 \mu\text{mole}$ of sodium oleate.

Enzymic Formation of U Factor—Data in Table III show that freshly prepared intact mitochondria or sonically disrupted mitochondria contain very little or no U factor activity when extracted and assayed according to the procedures described above. However, when such preparations are incubated for 1 to 2 hours at 37° a large increase in extractable U factor activity occurs. Such formation of U factor activity is accompanied by a virtually complete loss of phosphorylation activity of the mitochondria, suggesting that formation of the factor causes uncoupling of the phosphorylation mechanism. Similar experiments in Table III show that U factor is formed on incubation of sonically disrupted mitochondria at about the same rate as in intact mitochondria. The formation of U factor does not occur, however, if the mitochondria are first heated for 5 minutes in a boiling water bath prior to incubation. The formation of U factor thus appears to be enzymic in nature. The formation of U factor in suspensions of sonically treated mitochondria is a function of pH, being optimum between pH 5.6 and 6.5, as shown by the data in Table IV.

Effect of Cofactors and Inhibitors—The rate of enzymic formation of U factor in sonically disrupted mitochondria is affected by the presence of various agents. Data in Table V show that the formation of U factor is not inhibited by 0.001 M sodium cyanide, 0.001 M sodium azide, 0.01 M arsenate, or 0.001 M CaCl_2 . On the other hand, 0.02 M sodium fluoride inhibited U factor formation 50 per cent and 0.001 M HgCl_2 inhibited it about 80 per cent.

The data in Table V also show that inorganic phosphate is apparently not a requirement for the formation of U factor. Similarly the presence of ATP, ADP, DPN, or Mg^{++} had no effect. However, it is highly significant that the presence of ATP and CoA during the incubation of the sonically disrupted mitochondria often greatly reduced the amount of U factor extractable with isooctane. The inhibition under these circumstances approached 80 per cent, particularly with short reaction periods. CoA added alone was generally ineffective. Oxidized CoA was ineffective even when added with ATP. The possible significance of these observations is discussed below.

Mitochondrial Components Required for Formation of U Factor—Some simple fractionation experiments were carried out to establish which mitochondrial components are involved in the formation of U factor. Intact mitochondria were incubated for 2 hours at 37° , chilled, subjected to sonic treatment for 15 minutes, and then centrifuged at $120,000 \times g$ for 30 minutes in the Spinco model L ultracentrifuge to separate the sonically disrupted mitochondria into a soluble supernatant fraction and an insoluble pellet, which was resuspended in 0.03 M KCl. Each fraction, as well as the unfractionated suspension, was separately extracted with isooctane and the location of U factor determined by the uncoupling assay. The data in Table VI show that U factor formed in intact mitochondria on aging is recovered almost entirely from the particulate residue which presumably consists of fragments of the mitochondrial membrane with only a small portion present in the soluble protein fraction, which in turn presumably originates largely in the mitochondrial matrix.

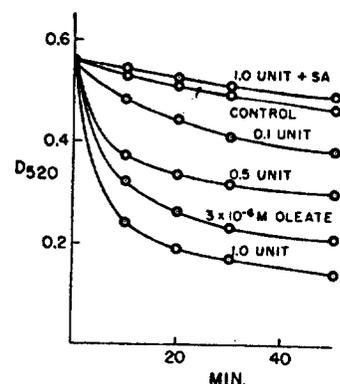


FIG. 1. Swelling action of U factor on rat liver mitochondria. Aliquots of isooctane solution of U factor were evaporated in matched cuvettes. A medium of 5.0 ml. 0.125 M KCl- 0.02 M Tris pH 7.4 was added to each tube. The effects of U factor were compared with the swelling action of $3 \times 10^{-6} \text{ M}$ sodium oleate. Optical absorbancy changes were measured at $520 \text{ m}\mu$ at 20° after the addition of washed rat liver mitochondria derived from 50 gm. whole liver (14). Serum albumin was added at 1.0 mg. per ml.

TABLE III
Formation of U factor and decline of P:2e ratio during incubation of mitochondria

In Experiment 1 each tube contained intact mitochondria isolated from 5.0 gm. whole rat liver, suspended in 10.0 ml. of 0.03 M KCl- 0.01 M Tris buffer pH 7.4. The tubes were incubated at 37° for 2 hours as shown. For analysis of U factor, the tubes were chilled in ice, the contents treated sonically for 15 minutes at 4° , and U factor extracted from the acidified contents with 3 equal volumes of isooctane and assayed as described in the text. Aliquots (1.0 ml.) of the mitochondrial suspension in parallel tubes were used to determine the change in the P:2e ratio as a result of the incubation at 37° . The test system for the latter determination contained 0.01 M β -hydroxybutyrate, 0.0024 M ADP, 0.03 M phosphate pH 7.4, 0.005 M MgCl_2 , 0.001 M DPN, 0.03 M glucose, 2.0 mg. Type V yeast hexokinase, and 0.03 M KCl in a total volume of 2.0 ml. Incubation was 20 minutes at 20° .

In Experiment 2, the details were the same except that the mitochondrial suspension was first treated sonically in 0.03 M KCl at 0° for 15 minutes prior to the 2 hour incubation at 37° ; tests of the P:2e ratio were not carried out.

Experiment No.	Type of preparation	U factor activity (units per tube)		P:2e	
		Before incubation	After incubation	Before incubation	After incubation
1	Intact mitochondria	2	55	2.20	0.32
	Intact mitochondria	2	50	2.16	0.22
	Boiled intact mitochondria*	2	2		
2	Sonic-treated mitochondria	5	60		
	Sonic-treated mitochondria	5	60		
	Boiled sonicated mitochondria*	5	5		

* Tubes were placed in boiling H_2O bath for 5 minutes before incubation at 37° .

Experiments were then carried out to determine the location of the enzyme capable of forming U factor. Freshly prepared sonically disrupted mitochondria were centrifuged and separated into a soluble fraction and a particulate residue. These fractions were incubated at 37° for 2 hours, separately or combined as

TABLE IV
Effect of pH on formation of U factor

Each tube contained sonic-treated mitochondria derived from 5.0 gm. of whole rat liver in 10.0 ml. 0.03 M KCl, buffered at pH 4.8 with 0.01 M acetate, at pH 5.6 and 6.5 with 0.02 M histidine, and at pH 7.4 and 8.5 with 0.01 M Tris. The tubes were incubated 1 hour at 37°, acidified, extracted with isoctane, and the U factor formed was determined as described in the text.

pH	U factor formed
	units
4.8	34
5.6	78
6.5	70
7.4	50
8.5	20

TABLE V
Effect of inhibitors and cofactors on formation of U factor

Assay carried out on sonic-treated mitochondria exactly as in Table IV, using a medium of 0.03 M KCl-0.01 M Tris buffer, pH 7.4. Additions to the medium were made as shown.

Additions	U factor formation
	units
1. None.....	105
0.001 M sodium cyanide.....	100
0.001 M sodium azide.....	95
0.01 M sodium arsenate.....	105
0.001 M calcium chloride.....	100
0.02 M sodium fluoride.....	50
2. None.....	70
0.001 M HgCl ₂	10
0.003 M ATP.....	65
0.003 M ADP.....	75
0.003 M DPN.....	75
0.001 M MgCl ₂	80
0.01 M phosphate.....	75
3. None.....	55
0.005 M ATP.....	45
0.005 M CoA-SH.....	50
0.005 M ATP + 0.005 M CoA-SH.....	10
0.005 M ATP + 0.005 M CoA (-SS-).....	40

shown, as was a sample of the original unfractionated suspension. The data in Table VI show that neither fraction possesses the entire activity of the whole suspension. The particulate fraction alone forms only a small amount of U factor, less than 15 per cent of that formed by the whole suspension. The soluble fraction alone shows only about 30 per cent of the activity of the whole suspension. When the two fractions were boiled before incubation, each lost its activity completely (Table VI). These findings indicate that the substrate and enzyme, or cofactors, presumably involved in the formation of U factor are not located in the same fraction.

When the supernatant fraction was boiled and incubated with unboiled particulate fraction, relatively little U factor was formed. Conversely, when the boiled particulate fraction was combined with unboiled supernatant fraction, full activity in the formation of U factor was restored. The experiment suggests that the particulate fraction contains a heat-stable sub-

stance, presumably a substrate or cofactor, necessary for the action of a heat-labile component in the soluble fraction in the formation of U factor. It must be pointed out that the heating of mitochondrial fractions as carried out above may cause changes in the extractability of U factor by isoctane, in addition to inactivation of a heat-labile factor.

Action of Serum Albumin on Phosphorylating Mitochondrial Fragments—Phosphorylating fragments of rat liver mitochondria prepared by the digitonin procedure (1, 10) lose phosphorylating activity on aging at 0°. Concomitantly, they also lose their activity in catalyzing the ATP-P_i³² exchange reaction and dinitrophenol-stimulated ATPase (18). Such inactivated preparations can be reactivated by the addition of serum albumin as shown in Table VII. It is seen that the addition of serum albumin reactivated almost completely the phosphorylation and exchange in a preparation of digitonin fragments aged for as long as 44 hours at 0°. Preparations aged for longer periods were not activated, but they had lost activity in oxidation of β-hydroxybutyrate because of the loss of bound DPN. These findings are thus similar to those of Pullman and Racker (7) on intact mitochondria.

Extraction of U Factor from Aged Digitonin Fragments—The reactivating effect of serum albumin on aged digitonin fragments suggested that U factor was being formed enzymically in the fragments during storage. This was confirmed by the extraction of fresh and aged digitonin fragments with isoctane, and assay of the extracts for uncoupling activity in a test system in

TABLE VI
Fractionation experiments on formation of U factor

In Experiment 1, 20.0 ml. of a suspension of rat liver mitochondria derived from a 12.0 gm. whole rat liver in 0.03 M KCl-0.02 M Tris, pH 7.4, was incubated 1 hour at 37°. It was then treated sonically 15 minutes at 4°. A 10-ml. aliquot was centrifuged at 105,000 × g. for 30 minutes to yield a supernatant fraction and the pellet, which was resuspended in 10 ml. 0.03 M KCl. After acidification, each fraction and the whole suspension was extracted with isoctane and assayed for U factor activity.

In Experiment 2, 10.0 ml. aliquots of freshly prepared sonic suspension, supernatant fraction, and resuspended residue, prepared as above and derived from fresh, unincubated mitochondria isolated from 6.0 gm. of whole rat liver, were incubated for 2 hours at 37°. Some of the tubes were first heated 5 minutes in a boiling H₂O bath before incubation, as indicated below.

Exp. No.	Fraction incubated	Fraction analyzed	U factor formed units
1	Intact mitochondria	Sonic-treated mitochondria.....	27
		Supernatant fraction.....	2
		Residue fraction.....	28
		Supernatant fraction plus residue.....	38
		Fraction incubated and assayed	
2	Whole sonic suspension	Whole sonic suspension.....	48
		Whole sonic suspension boiled.....	4
		Supernatant fraction.....	14
		Supernatant fraction boiled.....	2
		Residue fraction.....	10
		Residue fraction boiled.....	4
		Boiled residue + unboiled supernatant.....	64
Boiled supernatant + unboiled residue.....	12		

which freshly prepared digitonin fragments were employed. Data in Table VIII demonstrate that freshly prepared digitonin fragments contain a small but significant amount of material extractable with isoctane and capable of uncoupling phosphorylation. Extracts of the fragments aged at 0° for increasing periods of time contained increasing amounts of the uncoupling factor; the change was 9-fold over 72 hours at 0°. The uncoupling action of this factor was completely prevented by adding serum albumin to the test medium at levels of 2.0 to 5.0 mg. serum albumin per ml. That the formation of U factor in aging digitonin fragments is enzymic was shown by an experiment with boiled digitonin fragments in which no increase in isoctane-extractable, uncoupling factor occurred during incubation over 72 hours at 0°. Further evidence for the identity of the factor formed in aged digitonin fragments was obtained by washing aged fragments with serum albumin solution. The serum albumin solution was then extracted with isoctane which was found to contain the uncoupling factor.

These experiments show that the phosphorylating digitonin fragments contain the components necessary for the formation of U factor, although only a small fraction of the total capability of intact mitochondria in forming the factor is present.

DISCUSSION

The evidence summarized in this paper indicates that a potent uncoupling agent of oxidative phosphorylation is formed in intact mitochondria or in mitochondrial fractions on incubation in the absence of oxidizable substrates or adenine nucleotides by an enzymic reaction in which the substrate or a heat-stable cofactor is contributed largely by the insoluble particulate fraction, presumably derived from the membranes, and a heat-labile component is contributed largely by the soluble fraction.

The properties of the uncoupling agent suggest that it may be a long-chain fatty acid. The U factor is heat-stable and readily extractable from aqueous phases by isoctane, diethyl ether, or benzene, at pH 6.5 or below, but not extractable above pH 8. It is already known that fatty acids uncouple oxidative phosphorylation (19) and inhibit certain processes dependent on it, such as fatty acid oxidation (20). In addition fatty acids have profound effects on ATPase activity (19). Enzymic formation of free fatty acids from endogenous lipides of mitochondria appears to be a possible mechanism for the formation of an endogenous uncoupling agent, since the mitochondrial fraction of liver homogenates is known to have some phospholipase activity and the mitochondrial membranes or their fragments contain large amounts of phospholipide (*cf.* (21)), which could offer considerable potential substrate for the formation of free fatty acid. Lastly, identity of U factor as a fatty acid is suggested by the ability of serum albumin to prevent its uncoupling action; serum albumin also prevents uncoupling by fatty acids such as oleate presumably because of its avid capacity to bind fatty acids.

Additional suggestive evidence that U factor may be a fatty acid comes from the finding that the presence of ATP and CoA inhibits its enzymic accumulation. Mitochondria contain activating enzymes that cause the formation of fatty acyl-CoA esters from free fatty acids (22). Enzymic conversion of free fatty acids into the CoA esters could be expected to result in a form of the fatty acid which is more soluble in water and less readily adsorbed, and thus less inhibitory to phosphorylation. Furthermore, the CoA esters would not be expected to pass into isoctane from an aqueous phase. Enzymic formation of the

TABLE VII

Reactivation of aged digitonin fragments by serum albumin

A suspension of digitonin fragments (9) in distilled H₂O (1.10 mg. N per ml.) was aged at 2°. Aliquots were taken at 0, 24, and 44 hours and assayed for phosphorylation efficiency and the ATP-P_i³² exchange in the presence and absence of crystallized bovine serum albumin at a level of 2.0 mg. per ml. Assay details are described in the text.

Age of digitonin fragments	ATP-P _i ³² exchange, ATP formed		Oxidative phosphorylation P:2c	
	-SA*	+SA	-SA	+SA
	<i>μ</i> moles	<i>μ</i> moles		
hrs.				
0	122	148	1.98	2.16
24	44	154	1.08	1.92
44	5	124	0.14	1.64

* SA, serum albumin.

TABLE VIII

Formation of U factor on aging digitonin fragments

A suspension of digitonin fragments in H₂O (1.24 mg. N per ml.) was aged at 2°. Aliquots, 4.0 ml., were taken at the times shown, acidified, extracted with isoctane, and U factor activity of the extracts assayed.

Age of digitonin fragments	U factor per mg. enzyme N
hrs.	<i>units</i>
1	2
24	8
48	12
72	18
72 (boiled)	1

relatively less "toxic" CoA esters from the toxic free fatty acids may be at least partly responsible for the observation of McMurray and Lardy (17) that additions of CoA-SH increase or stabilize the P:2c ratio in suspensions of mitochondrial fragments obtained by sonic treatment of mitochondria. This effect of ATP + CoA-SH on the formation of U factor was somewhat variable and never produced complete inhibition of U factor formation, however.

The factor described here may be identical with the active principle of the mitochondrial uncoupling material described by Pullman and Racker (7) and may also be present in the mitochondria of Polis and Shmukler (8). The uncoupling activity of both substances is reversed by serum albumin. Furthermore Hülsmann *et al.* (9) have recently shown that the uncoupling activity of mitochondria preparations may be extracted with isoctane. The latter authors also showed that this factor inhibited the ATP-P_i³² exchange reaction. Very rapid formation of U factor from endogenous precursors may also be suggested as the basis for the finding that serum albumin, or one of a few other proteins, (23) must be present in the test medium in order to demonstrate any oxidative phosphorylation at all in suspensions of fly muscle mitochondria (23). Recent experiments of Wojtezak and Wojtezak (24) have shown that serum albumin soluble extract a benzene-soluble uncoupling agent from *Trichoplax* larval mitochondria.

Although it would appear simplest to conclude that the formation of U factor is a nonspecific consequence of the disruption of

cell or mitochondrial structure and subsequent lipolysis to produce free fatty acid, and that this mechanism has no particular physiological significance, such a conclusion seems premature. It appears necessary first to establish the identity of U factor with more certainty, and further work is now being carried out to purify the isooctane-extracted, mitochondrial factor by chromatographic means. Although many long-chain fatty acids have uncoupling activity, it is possible that the bulk of the endogenous U factor activity may be contributed by one specific type of fatty acid which may not be one of the more common or abundant fatty acids of animal tissues. Also, free or unesterified fatty acids are normally found in the blood and tissues as a transport form in significant concentrations (25). Furthermore, a physiological uncoupling function of free fatty acids has been postulated by Langdon to explain some features of the metabolic integration of fatty acid and pyruvate oxidation in mitochondria (26).

Promotion of mitochondrial swelling by U factor suggests that it is an important endogenous or intracellular factor in swelling, together with the other intracellular swelling agents, such as thyroxine, Ca^{++} , phosphate, and glutathione (*cf.* (27)). It is significant that serum albumin causes substantial potentiation of the action of ATP and Mg^{++} in reversing mitochondrial swelling (27), suggesting that the serum albumin combines with U factor.

SUMMARY

Incubation of intact or sonically disrupted rat liver mitochondria at 37° causes the loss of the ability to couple phosphorylation to respiration and the simultaneous formation of an isooctane-extractable, heat-stable uncoupling agent designated

as U factor. This factor also stimulates respiration in the absence of a phosphate acceptor system, inhibits the adenosine triphosphate (ATP)-inorganic orthophosphate- P^{32} exchange reaction, and stimulates ATPase activity. U factor also causes swelling of rat liver mitochondria. These actions of U factor are abolished by the presence of serum albumin.

Enzymic formation of U factor in sonically disrupted mitochondria is heat-labile and has an optimal pH of about 6.0. It is not affected by inhibiting respiration with cyanide or azide, by the addition of ATP, adenosine diphosphate, Mg^{++} , Ca^{++} , diphosphopyridine nucleotide, or arsenate, but is inhibited by fluoride and Hg^{++} . The presence of both ATP and the reduced form of coenzyme A greatly inhibits the accumulation of isooctane-extractable U factor, and this may account for some of the beneficial action of coenzyme A in oxidative phosphorylation.

The enzymic formation of U factor requires a heat-stable component, possibly a lipid, which is present largely in the particulate fraction from sonic-treated mitochondria, plus a heat-labile component which is present in the soluble fraction of sonic-treated mitochondria. Enzymic formation of U factor also occurred in phosphorylating digitonin fragments derived from mitochondrial membranes.

U factor has properties consistent with those of higher fatty acids and its enzymic formation from endogenous lipides may be responsible for the rapid inactivation of oxidative phosphorylation that occurs when mitochondria are disrupted. A physiological role for U factor appears possible.

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Carcinogenicity of Heated Fats and Fat Fractions¹

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SUMMARY—Two vegetable fats commonly used in commercial and home deep-fat frying of foods, and fractions thereof, were tested for carcinogenicity in NIH Black rats. The following fractions of fresh and heated (used repeatedly for deep-fat frying) hydrogenated vegetable fat and corn oil were prepared and tested: urea adduct, nonurea adduct, nonsaponifiable fraction, and volatiles. Intramuscular injections of the nonurea adducts of fresh and heated hydrogenated fat and of corn oil, repeated every 2 weeks for 14–17 months, induced sarcomas at the site of injection in NIH Black rats. The incidence was higher in rats receiving the nonurea adducts from corn oil than from hydrogenated fat, and higher also in the nonurea adducts from heated than from fresh fats. Few tumors, except papillomas of the forestomach, were induced in the alimentary tract or at other sites in 426 rats by the daily feeding for 17 months of fresh and heated fats and the urea and nonurea adducts of corn oil, with and without added benzo[*a*]pyrene. No subcutaneous or other tumors were induced in 864 rats given as newborns, a single subcutaneous injection of fresh corn oil, nonurea adducts of fresh and heated hydrogenated fat and corn oil, or the nonsaponifiable fractions and volatiles of these two cooking fats. Lymphosarcomas, sarcomas of the uterus, fibroadenomas of mammary gland, squamous-lined cysts of the forestomach, and a variety of other tumors were obtained in all groups of animals, but the incidence was comparable to that occurring spontaneously in older rats of the NIH Black line.—*J Nat Cancer Inst* 42: 275–287, 1969.

HEATED FATS have long been suspected as environmental carcinogens because incomplete combustion of many organic materials produces carcinogens. The experimental work on the carcinogenicity of heated fat was carefully reviewed by Arffmann (1). In 1939, Roffo (2) reported the induction of gastric adenocarcinomas, hepatic sarcomas, and papillomas of the forestomach in rats fed fat heated to 350°C for 30 minutes. Other

workers, however, could not confirm Roffo's results and were unable to obtain tumors of the glandular stomach, though they did find papil-

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omas of the forestomach (3-5). Beck and Peacock (3) attributed papillomatosis to a toxic factor in the heated fat which interfered with the absorption and metabolism of vitamin A. They demonstrated a decrease in vitamin A in the livers of rats fed heated fats and showed that a diet rich in vitamin A inhibited papillomatosis of the forestomach.

Local sarcomas have been induced in rats at the site of subcutaneous injections of heated and unheated fats (6-9). The active substance in these cases does not appear to be benzo[a]pyrene or related compounds (10) and has not been demonstrated on oral administration, except possibly through the mechanism of vitamin A depletion and subsequent papillomatosis of the forestomach.

Perkins and Kummerow (11), studying the chemical changes in heated fats, found that treatment with urea resulted in separation of the unchanged fatty acids from oxidized material. Sixty-four percent of the fatty acids formed urea adducts (UA's) and the remaining 36% failed to form complexes with urea [non-urea-adduct (NUA) fraction]. The UA-forming fatty acids appeared to be free of polymers and to be composed of a mixture of normal straight-chain fatty acids. The NUA-forming fraction contained some linoleic and oleic acids but also a considerable amount of polymeric compounds of high molecular weight, with a high percentage of hydroxyl and carbonyl groups.

In the experiments reported here, fresh and repeatedly heated fats, and several chemical fractions thereof, were assayed for carcinogenic activity. Newborn rats received single subcutaneous injections and weanling and adult rats received repeated intramuscular injections of the fats or oral administration in the diet.

MATERIALS AND METHODS

Fresh hydrogenated fat and corn oil were purchased from commercial sources through the General Services Administration of the Federal Government. Used or heated hydrogenated fats were discards of a local cafeteria that obtains its cooking fats from the same source. Fresh corn oil was heated in the laboratory in 8-9 kg lots at 200°C for 6-8 hours a day for approximately 50 hours, or until an iodine number of <87 [Hanus method

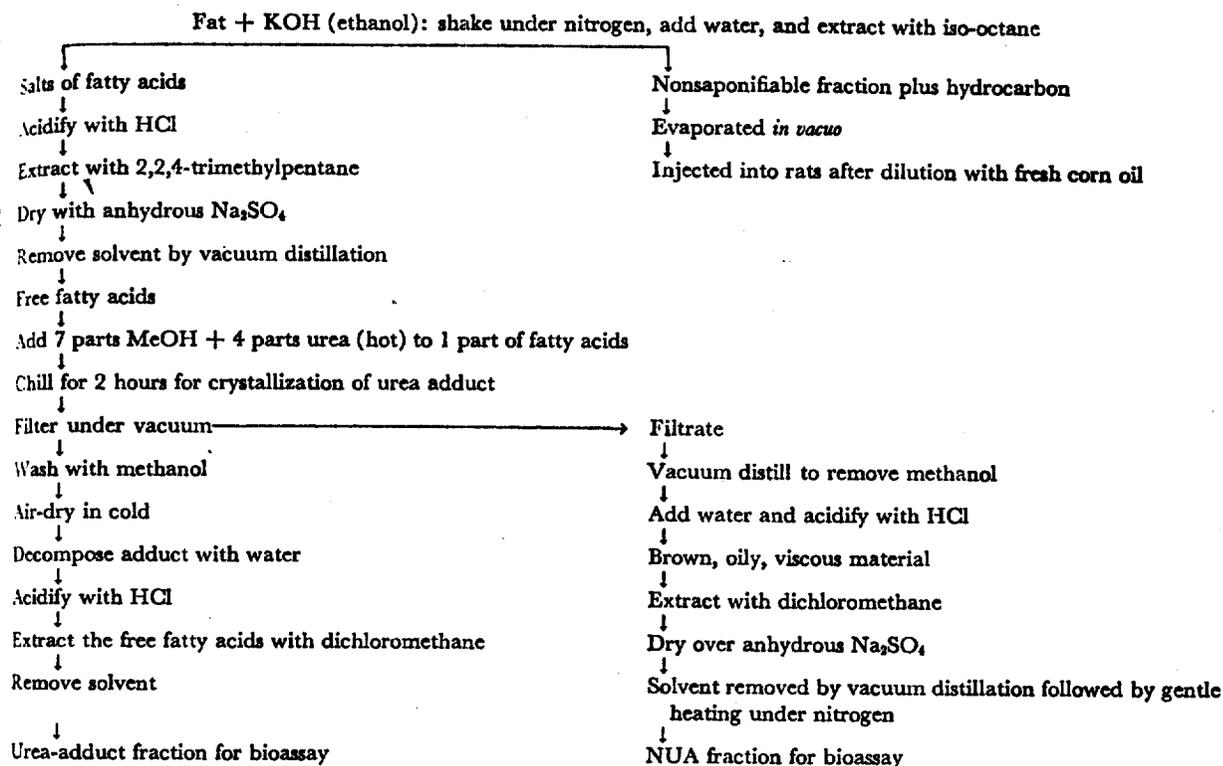
(12)] and a refractive index of 1.4812 or greater was obtained. During heating, the fat was aerated at the rate of 250 cc/min/kg oil by air blown through a fritted glass tube. From time to time peeled, sliced potatoes were fried in the oil to simulate home and commercial deep-fat frying. After completion of the heating procedure, the fat was stored at 4°C until use.

The procedure for fractionation of the fats is outlined in chart 1. A 135 g portion of fat was added to an 8-liter flask containing 44 g KOH dissolved in 500 ml ethanol. The flasks were flushed with nitrogen to remove the air and to prevent further oxidation and then placed for about 30 hours in a wrist-action shaker. Preliminary tests had shown that this was adequate to assure complete saponification. The reaction mixture was diluted with water to solubilize the potassium salts of the various fatty acids, and the nonsaponifiable fraction (principally sterols and any hydrocarbon material that might have formed) was extracted with 2,2,4-trimethylpentane (iso-octane). The iso-octane containing the nonsaponifiable fraction was then evaporated by vacuum distillation. The nonsaponifiable fraction usually was obtained as a noncrystalline, syrupy mixture, which was diluted with fresh corn oil for injection into rats. However, occasionally a crystalline substance was obtained; further purification showed it was B-sitosterol.

The saponified fraction was acidified with HCl; the free fatty acids were extracted with 2,2,4-trimethylpentane and dried with anhydrous sodium sulfate, and the solvent was removed by vacuum distillation. The UA fraction was prepared from the free fatty acids; 1 part of free fatty acid was added to a hot solution of 7 parts methyl alcohol and 4 parts urea. The mixture was then chilled for a minimum of 2 hours to allow for complete crystallization of the UA, filtered under vacuum in the cold, washed several times with cold methanol, and air-dried in the cold. The adduct was then decomposed with water and acidified slightly, and the free fatty acids were extracted with dichloromethane (methylene chloride). Removal of the solvent yielded the normal free fatty acids capable of forming UA's. The UA fractions were all white solids at room temperature.

The filtrate containing the NUA fraction was vacuum-distilled to remove the methanol. Water

CHART 1.—Outline of procedure for chemical fractionation of fat



The NUA fraction of corn oil heated at 200°C for 50 hours averaged 42% of the total sample (range: 31–45%). In one sample the NUA fraction was 36.60% and the UA fraction, 53.05%. The remaining 10% was mostly glyceride. Analysis of one sample of hydrogenated fat, heated under the same conditions, yielded only 5% NUA fraction. See also the analysis of fresh corn oil and of corn oil heated to 200°C for 24 hours in Appendix 1.

was added and the mixture acidified with HCl. A brown, oily, viscous substance was obtained, which was extracted with dichloromethane and dried with anhydrous Na_2SO_4 . The solvent was then removed by vacuum distillation, followed by gentle heating in an evaporating dish with nitrogen blowing over the surface to remove the last trace of solvent. The dark, oily, viscous material remaining contained the fatty acids that had been changed by heat and/or oxidation, and could no longer form adducts with urea. This NUA fraction contained polymerized material and any cyclic and branched chain compounds formed in the heating process.

Two volatile fractions were prepared from corn oil by distillation: One fraction contained the material that distilled over in heating for 5 hours at 100°C, the other the material that distilled over during an additional 10 hours of heating at 200°C.

The distillates were collected in ethanol cooled in a dry-ice bath.

Biological assay.—The randombred NIH Black rats used for bioassay were obtained from the Animal Production Section of the National Institutes of Health. This strain was chosen because it has a low incidence of spontaneous neoplasms, except for lymphomas (6%), and of uterine neoplasms (3.3%) and fibroadenomas of the breast (2.5%) in old females (13). Ten males or fifteen females were housed together in large rabbit cages. The animals were allowed free access to rat pellets (Ralston-Purina Co., Wilmington, Del.) and water. Because of limitations of space, animals, and materials, not all fractions were tested by every route.

Forty to sixty newborn rats were given a single subcutaneous injection of one of the following materials: fresh whole corn oil, the UA and NUA

of heated corn oil, the NUA's of fresh or heated hydrogenated fat, the nonsaponifiable fraction of fresh or heated corn oil or hydrogenated fat, and 2 volatile fractions of heated corn oil. The material was injected into the subcutaneous tissue at the nape of the neck in volumes varying from 0.01–0.05 ml/rat. The infants were then returned to their mother. They were weaned and sexed at 30 days of age, and housed in groups of 10 or 15 according to sex.

The following materials were assayed orally: fresh and heated whole hydrogenated fat and fresh whole corn oil, UA and NUA of heated corn oil, and the dregs of heated hydrogenated fat. These fractions (except for the dregs) were tested alone and with added benzo[*a*]pyrene. The test materials were mixed in ground rat chow and prepared fresh weekly. The fresh and heated hydrogenated fats were incorporated into the diet as a 20% mixture, and the corn oil as a 10% mixture. The UA and NUA of corn oil were given as 5% adduct plus 5% fresh corn oil. The rats were fed these diets for 17 months and then a regular diet of rat pellets for the rest of their lives. Once a week each rat was given 2000 U vitamin A, 400 U vitamin D, and 5 mg vitamin E (in corn oil). Benzo[*a*]pyrene was freshly mixed in the vitamin supplement fed to one-half the rats in each group, at a dose level of 0.5 mg/rat. For each test material, 30–40 weanling males and 50–60 weanling females were used. Daily food consumption was comparable for all the diets, and weight gains were satisfactory for rats on all diets except the NUA of heated corn oil, where weight gain was slightly retarded.

The following materials were assayed intramuscularly: fresh whole corn oil, fresh and heated whole hydrogenated fat, the NUA's of fresh and heated hydrogenated fat and corn oil, and the volatiles of heated corn oil (200°C for 10 hr). Twenty male and 20 female weanlings were used to assay each material tested except the volatiles, where only 8 male and 8 female weanlings were used. The material was injected into the right thigh muscle at 2-week intervals for 15 months and was contained in a volume of 0.1 ml, except for the volatiles of corn oil. Because of the local necrotizing properties of these volatiles, only 0.01–0.04 ml of this fraction was given per injection. The whole fresh and heated hydrogenated fats

were assayed in rats given injections as newborns of the NUA of fresh and heated hydrogenated fat. These animals were 8 months old when the intramuscular injections were begun, and appeared healthy and tumor-free at that time.

RESULTS

No tumors were induced at the injection site in 864 rats receiving a single subcutaneous injection of fat or fat fraction as newborns. The percent of lymphosarcoma varied from zero in animals given the nonsaponifiable fractions to 5.5 in rats given NUA's. Three of them developed fibroadenomas of the mammary gland and ten developed miscellaneous tumors. The incidences of lymphosarcoma and mammary fibroadenoma observed in these rats are comparable to those occurring spontaneously in the NIH Black line. Hueper (13), for example, reported an apparent spontaneous incidence of 6% lymphosarcoma and 2.5% of mammary fibroadenomas in 19- to 20-month-old NIH Black rats given injections of hypertonic sugar solutions. Hence it seems unlikely that any of these tumors were induced.

Many of the rats receiving volatile fractions of heated corn oil developed marked deformities of the spine, probably from a toxic effect of these materials on the developing vertebrae. Several rats in different experimental groups had submucosal cysts of the forestomach. These cysts were lined by squamous epithelium and filled with keratin, and were usually at the limiting ridge between the glandular stomach and the forestomach (figs. 1 and 7). Although these cysts have not been described previously in NIH Black rats, they are believed to be spontaneous in origin.

Several fat fractions were incorporated into the diet and fed daily to the rats for about 17 months. One-half the animals in each group also were fed 0.5 mg benzo[*a*]pyrene once a week, mixed in the vitamin supplement. Table 1 summarizes the data. Papillomas of the forestomach occurred predominantly in males—in 9 of 185 males versus 3 of 20 females. In addition, 3 rats had squamous carcinoma of the forestomach, 1 had squamous carcinoma of the esophagus, 1 had an adenocarcinoma of the cecum, and 7 had non-neoplastic squamous cysts of the forestomach. Of the 17 neoplasms in the

alimentary tract, 10 occurred in rats given benzo[*a*]pyrene weekly and must be attributed, at least partly, to the effect of the carcinogen. However, 2 of the squamous carcinomas of the forestomach and 5 of the papillomas were in rats that had not received benzo[*a*]pyrene. The number of lesions in the alimentary tract of rats fed fats and fat fractions is somewhat greater than we obtained in rats given these materials subcutaneously or intramuscularly, but is probably not significantly greater than is often found in old rats examined carefully for tumors at autopsy.

Thirty of the neoplasms which arose in rats on the oral regimens were outside the alimentary tract. Seven rats had fibroadenomas of the mammary gland, five had lymphosarcomas, five had adenocarcinomas of the uterus, and two had reticulum cell sarcoma. A variety of other tumors were found, but only in one rat in each group. There is no evidence that any of these extra-alimentary-tract tumors were induced by feeding of the fat or fat fractions.

Table 2 summarizes the data on rats given intramuscular injections of fresh and heated hydrogenated fat and fresh corn oil, the NUA's of these materials, and the volatiles (100°C for 5 hr) of corn oil. The fat and fat fractions were injected into the right hind thigh muscles every 2 weeks for 15 months. Thirty-one sarcomas were induced at the site of injection by the NUA's. The incidence of sarcomas was higher in the adducts prepared from heated hydrogenated fat (21%) and heated corn oil (59%) than from the NUA's of fresh hydrogenated fat (6%) and fresh corn oil (14%). Corn oil fractions were about three times more effective in inducing sarcomas than hydrogenated fat. No local tumors were obtained in rats given intramuscular injections of whole fresh and heated hydrogenated fat or fresh corn oil, or the volatiles of corn oil. These results suggest that fresh hydrogenated fat and corn oil contain an ineffective quantity of carcinogen which can be extracted and concentrated in the NUA fraction. Corn oil either contains a larger quantity of the carcinogen or a more potent carcinogen than hydrogenated fat. The activity or quantity of this carcinogen is apparently increased by repeated heating of the fats in the frying of foods.

Many local tumors were fibrosarcomas (figs. 2, 4, and 5), but others appeared to be a mixture of rhabdosarcoma and fibrosarcoma. Six sarcomas had metastasized to lungs, lymph nodes, or perirenal tissue. In general, the tumors were slow-growing and were usually allowed to grow for several weeks before the animal died or was killed. At autopsy, they averaged about 4 cm in largest diameter (range: 0.5–6.0 cm). The earliest tumor was in a rat killed after 9 months of treatment, and the latest in rats killed at the termination of the experiment (20 months after the start of treatment).

In addition to local sarcomas, 20 other primary neoplasms were obtained in the 243 rats given intramuscular injections of fat and fat fractions. Seven rats had lymphosarcomas, three had carcinomas of the uterus, three had papillomas of the forestomach, two had fibrosarcomas of the abdominal cavity (figs. 3 and 6), and five other neoplasms occurred in a single rat each. Except for the fibrosarcomas and the papillomas, this is about the reported spontaneous incidence of these tumors in the NIH Black rats (13, 14) and agrees with our previous experience with this line.

DISCUSSION

The induction of sarcomas at the site of intramuscular injections of the NUA's of fresh and heated hydrogenated vegetable fat and corn oil indicates a carcinogen is present in these materials. Corn oil appears to contain either more of the carcinogen or a more potent one than hydrogenated fat. The greater activity shown by the NUA's of heated fats as compared with similar fractions prepared from fresh fats apparently resulted from the process of heating and cooking. Several changes occur during heating (15). Fats undergo autoxidation at temperatures less than 100°C, while, at 200–300°C, thermal polymerization takes place in the absence of oxygen, and thermal oxidation occurs in the presence of oxygen. Formation of conjugated hydroperoxides, cyclic compounds, and polymers may result from autoxidation. Thermal polymerization of an unsaturated fat may produce cyclic monomers, dimers, and polymers, while thermal oxidation can cause an increase in conjugation of the fat and a decrease

TABLE 1.—Tumors obtained in NIH Black rats fed cooking fats and fat fractions

Material	Dose % of diet	Number of rats*	Sex	Median age at death (months)	Range (months)	Tumors of alimentary tract (age in months)†	Other tumors (age in months)†
Fresh whole hydrogenated fat	20%	21	F	23	6-23	0	Reticular cell sarcoma (17) Adenocarcinoma of uterus (23) Fibroadenoma of breast (19)
		19	M	12	10-12	0	—
Fresh whole hydrogenated fat + benzo[a]pyrene	20% 0.5 mg/wk	21	F	23	7-23	0	Fibromyxosarcoma of pelvis (20) Adenocarcinoma of endometrium (23) Fibroadenoma of breast (23)
		17	M	23	6-23	Squamous cyst of F.S.	Fibromyxosarcoma of neck (23) Lymphosarcoma of abdomen (14) Adenocarcinoma of ureter (23)
Heated whole hydrogenated fat	20%	27	F	22	11-23	0	Rhabdomyosarcoma of abdominal cavity (15) Reticulum cell sarcoma (17)
		21	M	21	7-23	2 papillomas of F.S. (21)	Squamous carcinoma of nasal cavity (19)
Heated whole hydrogenated fat + benzo[a]pyrene	20% 0.5 mg/wk	29	F	22	10-23	2 papillomas of F.S. (22-23) 2 squamous cysts of F.S.	Lymphosarcoma (11)
		20	M	22	11-23	Papillomas of F.S. (21)	Subcutaneous fibrosarcoma (23)
Fresh whole corn oil	10%	28	F	23	11-23	0	Adenocarcinoma of endometrium (23)
		19	M	12	6-12	2 papillomas of F.S. (12) Squamous cyst of F.S.	—
Fresh whole corn oil + benzo[a]pyrene	10% 0.5 mg/wk	26	F	22	12-23	Papilloma of F.S. (22)	Two fibroadenomas of breast (12 and 19)
		18	M	22	11-23	Squamous carcinoma of F.S. (21)	Mesothelioma of heart (20) Lymphosarcoma (21)
Urea adduct of heated corn oil and fresh whole corn oil	5% 5%	22	F	20	10-24	2 squamous carcinomas of F.S. (24)	Fibroadenoma of breast (24) Adenocarcinoma of endometrium (22) Granulosa cell carcinoma of ovary (24)
		18	M	22	9-24	Squamous cyst of F.S.	Adenoma of adrenal cortex (24) Lymphosarcoma of abdominal nodes (22)

Nonurca adduct of heated corn oil and fresh whole corn oil + benzo[a]pyrene	5% 5% 0.5 mg/wk	27	F	21	9-23	0	Two fibroadenomas of breast (22 and 23) Adenocarcinoma of pancreas with metastases (23) Lymphosarcoma of abdominal nodes (13) Fibrosarcoma of salivary gland (21)
		17	M	23	11-23	Squamous carcinoma of esophagus (11) Papillomas of F.S. (21) Adenocarcinoma of cecum (23) Squamous cyst of F.S.	
Nonurca adduct of heated corn oil and fresh whole corn oil	5% 5%	23	F	10	9-10	0	— —
		15	M	10	6-24	Papilloma of F.S. (9)	
Nonurca adduct of heated corn oil and fresh whole corn oil + benzo[a]pyrene	5% 5% 0.5 mg/wk	28	F	22	6-24	0	Adenocarcinoma of endometrium with metastases (22) —
		13	M	23	20-24	2 papillomas of F.S. (20 & 21) Squamous cyst of F.S.	
Dregs of heated hydrogenated fat	20%	5	F	12	12	0	— —
		8	M	10	8-12	0	
TOTAL.....		442					

*Only rats 6 months of age or older are included.

†The first number indicates the number of rats (if more than one) with a specific tumor. Papillomas of the forestomach (F.S.) are solitary unless designated as multiple by an "s."

TABLE 2.—Tumors obtained in NIH Black rats given biweekly intramuscular injections of cooking fats and fat fractions

Material	Dose (ml)	Number of rats*	Sex	Median age at death (months)	Range (months)	Sarcomas at injection site	Ages at time of first and last tumors	Other tumors and related lesions
Fresh whole corn oil	0.1	17	F	22	11-22	0	—	Hyperplastic nodule of liver Hyperplastic nodule of liver Papilloma of forestomach Lymphosarcoma of abdominal lymph nodes
	.1	14	M	22	20-22	0	—	
Fresh whole hydrogenated fat†	.1	16	F	12	12-23	0	—	Adenocarcinoma of endometrium Squamous cyst of forestomach
	.1	7	M	14	14	0	—	
Heated whole hydrogenated fat†	.1	23	F	14	14-23	0	—	Lymphosarcoma of kidney and ovary; Squamous cyst of forestomach Fibroadenoma of breast Papilloma of forestomach
	.1	14	M	14	12-14	0	—	
Nonurea adduct of fresh hydrogenated fat	.1	17	F	22	13-22	2	22	Cystadenoma of bile duct Squamous cyst of forestomach Lymphosarcoma, abdominal and thoracic lymph nodes
	.1	17	M	13	6-22	0	—	
Nonurea adduct of heated hydrogenated fat	.1	20	F	16	9-23	6‡	11-21	Two fibrosarcomas of abdominal cavity Adenocarcinoma of colon Squamous carcinoma of ovary Two lymphosarcomas of ileocecal region
	.1	18	M	18	7-22	2	16-20	
Nonurea adduct of fresh corn oil	.1	19	F	22	7-22	4	21-22	Lymphosarcoma, generalized
	.1	18	M	22	8-22	1	22	
Nonurea adduct of heated corn oil	.1	13	F	19	11-22	9‡	15-20	— Lymphosarcoma of abdomen Papillomatosis of forestomach and ulcers
	.1	14	M	16	12-22	7	15-22	
Volatiles of corn oil heated at 200° C	.04	8	F	23	6-23	0	—	Squamous carcinoma of cervix uteri Adenocarcinoma of endometrium
	.01-.02	8	M	23	13-23	0	—	
TOTAL.....		243				31		

*Only rats 6 months of age or older are included.

†Originally given a single subcutaneous injection of nonurea adduct of fresh and heated hydrogenated fat. Intramuscular injections begun at the age of 8 months.

‡Three tumors in each of these groups had metastasized.

in unsaturation and in nonconjugated fatty acids, probably without formation of cyclic compounds. There is some evidence that unsaturated carbonyl compounds also may be produced. Thermal oxidation of fat reportedly decreases the amount of linoleic acid, probably through oxidation scission and polymerization of the double bonds in linoleic acid (11). Treatment with urea separates unchanged fatty acids from the oxidized material. Hence the UA contains normal straight-chain fatty acids, free of polymers, while the fraction that does not form adducts with urea contains a considerable amount of polymeric compounds of high molecular weight, along with small amounts of linoleic, oleic, and saturated fatty acids. The carcinogenic effect from the injection of heated fats appears to reside in the polymerized derivatives of altered fatty acids found in the NUA fraction.

Possibly the tumors were not induced by a chemical carcinogen but by physical influences analogous to the solid-state carcinogenesis of plastic and metal discs. The injected fat fractions are slowly absorbed from the local site, and residual deposits of the injected material can be found for a considerable time after the injections. However, the failure to obtain local tumors from the injection of fresh whole corn oil makes this possibility unlikely, and the fact that relatively few tumors were induced by NUA's of fresh fats, in contrast to the many induced by extracts of heated fats, makes it probable that the sarcomas were chemically induced.

Few tumors were induced by the oral administration of heated fats or fat fractions, either alone or in combination with added benzo[a]pyrene. Seventeen rats developed neoplasms of the gastrointestinal tract and seven, non-neoplastic squamous cysts. Twelve of the neoplasms consisted of single or multiple papillomas of the forestomach, of which seven arose in rats fed benzo[a]pyrene and five arose in an approximately equal number of rats not given the chemical carcinogen. Three rats had squamous cell carcinomas of the forestomach. The failure to induce an appreciable incidence of tumors in rats fed NUA's or given a single subcutaneous injection of these fractions indicates that repeated intramuscular injection is a more sensitive assay system for weak or minimal amounts

of carcinogens. However, Sugai *et al.* (16) reported that feeding rats the NUA of heated corn oil along with a noncarcinogenic level of 2-fluorenyl-acetamide induced tumors of the liver, ear duct, and mammary gland.

It was hoped that the present experiments would help to answer the question of whether ingestion of heated fats by man is a factor in the development of tumors of the gastrointestinal tract and other sites. However, except for papillomas of the forestomach, few tumors were obtained when various fractions of cooking fat were assayed orally. The only malignant tumors unequivocally induced were sarcomas at the site of repeated injections of the NUA's. Hence it is difficult to relate the results of the animal assays to the human situation. Perhaps the known differences in the anatomy of rodent and human stomachs make the rodent a poor choice for such studies. Possibly some other species, such as the dog or small primate, might give more meaningful results, though such studies are made difficult by the greater cost, limited availability of healthy stock of known genealogy, and the longer period of treatment and observation that would seem to be required by the longer lifespan of such animals. However, the present results do suggest the advisability of avoiding the excessive reuse of cooking fats in the preparation of food for human consumption.

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Appendix 1⁴

ASSAYS OF NON-UREA ADDUCT (NUA) FRACTION OF CORN OIL

Property	Fresh corn oil	Corn oil heated 24 hours at 200°C	NUA fraction
% NUA fraction	0.5*	17.5†	100
Molecular weight (after sap)	302*	543†	354‡
Iodine value	122	100	91.5
Peroxide value	1.0	2.0	9.5
Acid value	0.16	1.3	192
Sap value	186	200	221
Refractive index (25 c)	1.4730	1.4792	1.4879
Carbonyl value mg/kg	§	—	63
Oxirane value mg/kg		—	41

*Johnson, O. C., and Kummerow, F. A.: *J Amer Oil Chemists' Soc* 34: 407, 1957.

†Sahasrabudhe, M. R., and Bhalariao, V. R.: *Ibid* 40: 712, 1963.

‡Cryoscopic Detn, with *t*-butanol as the solvent.

§Modified Henick DNP technique.

||Pieric acid method.

GAS LIQUID CHROMATOGRAPHY

GLC examination of the fatty-acid methyl esters (transesterified with BF_3 -methanol) showed a normal distribution of corn-oil fatty esters except for a slightly lower than normal amount of stearate.

For this assay a DEGS column was used isothermally at 190°. Examination on an SE-30 column, programmed from 200–350°C, showed about 14.4% dimeric esters.

If the mixture were comprised of 14.4% dimer and the remainder monomer, the molecular weight would be 290 (using 254 equivalent weight determined by saponification). Since the molecular weight found was 353, it is apparent that some oligomers have been formed. If one assumes that the quantities of each decrease with increase chain length, the next most populous species would be the trimer. Assuming only mono, di, and trimers present, the composition would be 73.3, 14.4, and 12.3%, respectively, to give a molecular weight of 353.

⁴R. J. SIMS, A. P. BENTZ, and J. A. FIORITI, *General Foods Corporation, White Plains, New York.*

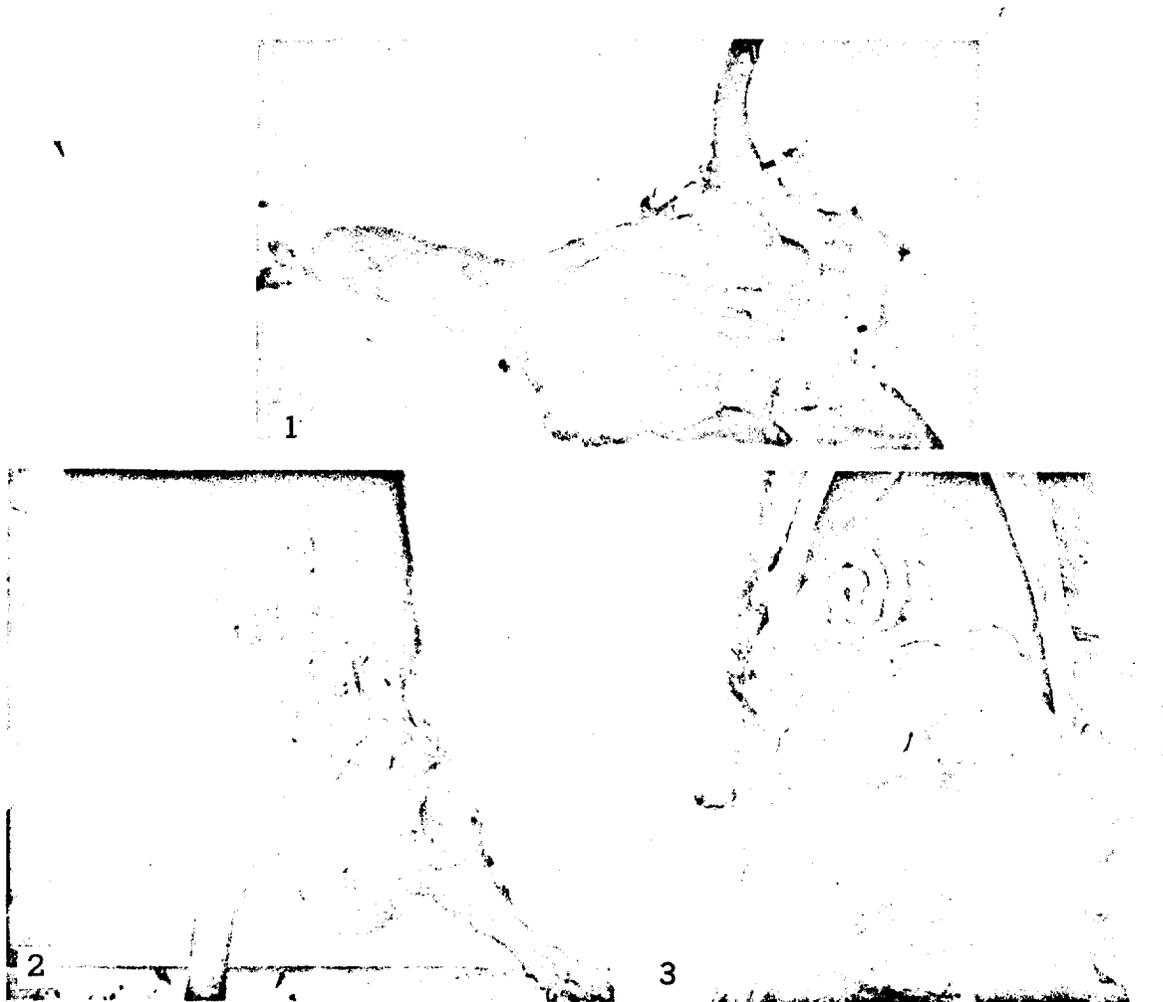


FIGURE 1.—Stomach of a 22-month-old rat given biweekly injections of the nonurea adduct (NUA) of fresh vegetable fat. A 0.5 cm nodule is on the limiting ridge between the glandular stomach (*left*) and the forestomach (*right*). Portion of esophagus is attached (*above*). About actual size.

FIGURE 2.—Sarcoma in the right thigh of a 17-month-old female rat given intramuscular biweekly injections of NUA of heated vegetable fat. Tumor measures 4.0 × 4.0 × 3.0 cm. About three-fourths actual size.

FIGURE 3.—A 16-month-old female rat given biweekly injections in the right thigh of NUA of heated vegetable fat. The dumbbell-shaped myxofibrosarcoma (9.0 × 5.0 × 3.0 cm) is partly in the abdominal cavity and partly in the left flank. No tumor was found at the site of injection (right thigh). About three-fourths actual size.

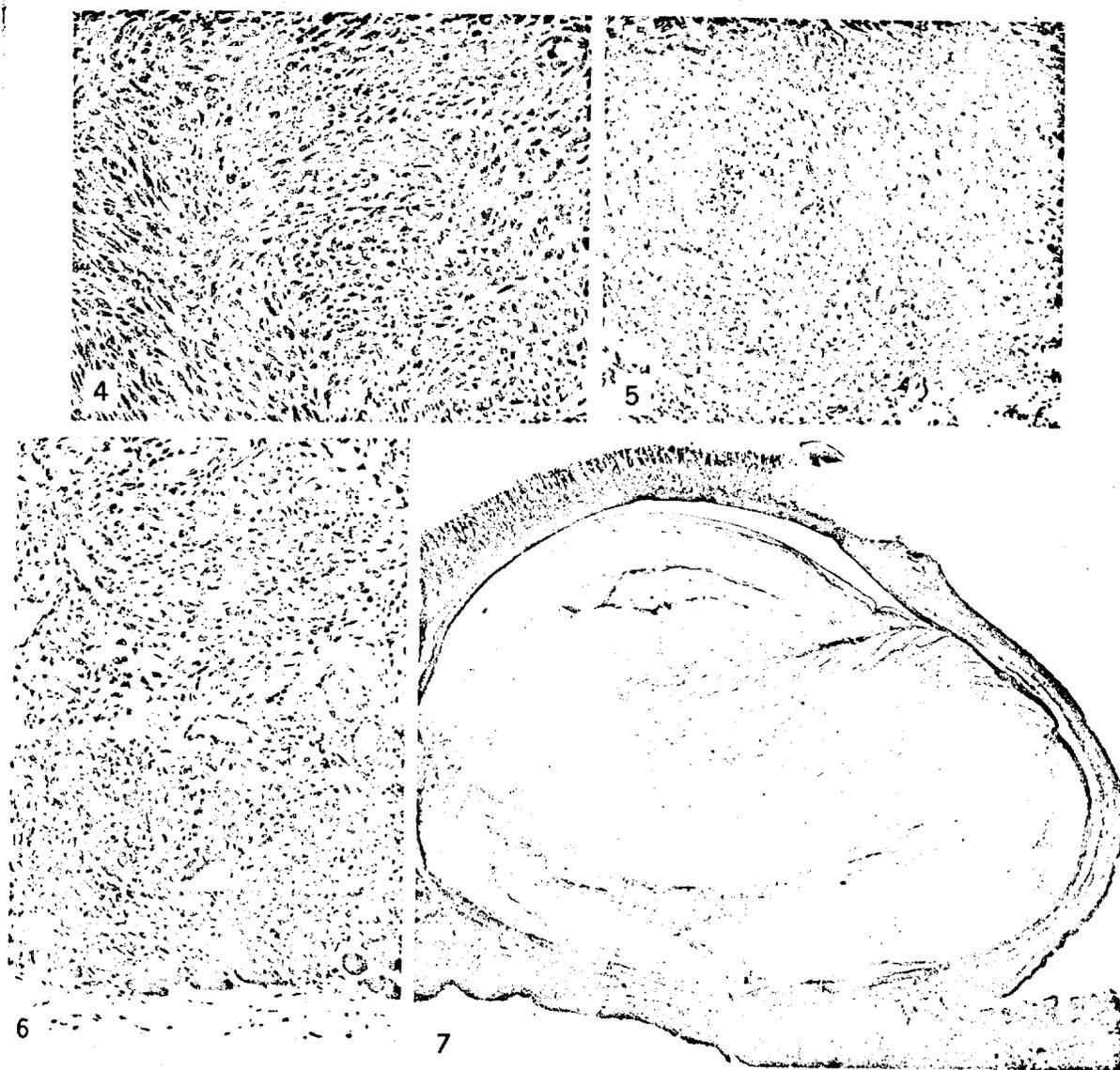


FIGURE 4.—Histologic appearance of tumor shown in figure 2. Cells are elongated but produce little or no collagen. Nuclei are often rounded on the ends. Cytoplasm is frequently ribbon-shaped and is reddish after Masson's trichrome stain. Cross striations were not seen. Hematoxylin and eosin. $\times 120$

FIGURE 5.—Lung of animal seen in figure 2. One of several metastatic foci is shown; all stained similarly to the primary lesion after Masson's trichrome procedure. Hematoxylin and eosin. $\times 120$

FIGURE 6.—Histologic appearance of the fibrous portion of the tumor shown in figure 3. Numerous whorls of fibroblasts producing a considerable amount of collagen are visible. Invasion of muscle of the abdominal wall (*below*). Hematoxylin and eosin. $\times 120$

FIGURE 7.—Histologic appearance of the nodule illustrated in figure 1. Between the glandular mucosa (*left*) and the forestomach (*right*) is a cyst lined by squamous epithelium and filled with keratotic material. Epithelium of the nodule is nowhere continuous with the epithelium of the forestomach. These cysts are considered to be spontaneous, arising probably from the squamous mucosa of the forestomach. Hematoxylin and eosin. $\times 25$

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Pharmacological Effects of Fatty Acids, Triolein and Cottonseed Oil.

By

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(Received March 8, 1961)

Various triglycerides in emulsion show marked pharmacological properties on intravenous administration. For example, several with low molecular weights have been found distinctly toxic on intravenous injection (WRETLIND 1957 b; ORÖ, WESTERHOLM & WRETLIND 1959). On the other hand, fats of higher molecular weights, such as cottonseed oil, triolein and certain vegetable fats, show little or no toxicity on intravenous injection into mice. Hence it is possible to produce injectable emulsions of cottonseed oil as a carrier of agents, such as higher fatty acids, that owing to insolubility could not otherwise be studied for their pharmacological effects. Such fatty acid emulsions have enabled us to determine the toxicity of fatty acids on intravenous injection.

In the same investigation we have also studied the effects of these emulsions on respiration and blood pressure in cats. It was found that emulsions containing only cottonseed oil or triolein could cause reduced blood-pressure and apnoea. These effects were characterised by such pronounced tachyphylaxis that frequently they were noticeable only on the first injection. They were readily distinguishable, therefore, from the affects of the different fatty acid emulsions in which cottonseed oil acted as carrier.

Lastly, we conducted experiments on guinea pig small intestine in order to study the effects of fatty acid emulsions on smooth muscle.

Experimental.

Animal Experiments.

For determinations of toxicity, mice weighing 13-34 g were used. The emulsions or solutions of fatty acids were injected into the tail vein. Each fatty acid was administered to at least six groups each of 10 animals. The calculations of LD50 with their standard

errors were by the method of MILLER & TAINTER (1944); the results are summarised in figure 1.

Some 100 cats weighing 2-6 kg were used for investigating the *respiratory* and *circulatory* responses to cottonseed oil, triolein and fatty acid emulsions. These animals were anaesthetized with diallylmal (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 electroencephalograph¹⁾ with supplementary detectors. A Statham pressure transducer, model P 23 AA²⁾, 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 % CaCl₂; 0.02 % MgCl₂ · 6 H₂O; 0.1 % NaHCO₃; 0.005 % NaH₂PO₄ · H₂O; and 0.1 % glucose). The organ bath was aerated with air. A Grass force-displacement transducer, model FT-03, was employed for recording the tension of the gut. In this connection we also studied the ability of fatty acids to abolish contractions elicited by histamine. For this purpose histamine (0.2 µg/15 ml) was first added to the organ bath and fatty acid emulsion was added without prior washing when maximal contraction had been obtained. The observed relaxation of the intestine was recorded.

Test Solutions and Test Emulsions.

For the *toxicity determinations* in mice the lower *fatty acids* (acetic, propionic, butyric, isovaleric, norvaleric, caproic and heptylic) were administered in aqueous solution, whereas the higher fatty acids, because of their insolubility in water, were emulsified. To prepare these *emulsions* the fatty acids were first dissolved in heated cottonseed oil, which served as carrier. Phosphatides, sodium cholate and glycerin monostearate were used as emulsifiers (table 1). The emulsions were homogenized in a Logeman apparatus (WRETLIND 1957 a). The pH of the solutions and emulsions was adjusted to 7.3 with NaOH.

The concentrations of fatty acids were so chosen that the injected volume required for determining LD50 amounted to between 3 and 45 ml per kg body weight. Thus acetic, propionic, butyric, isovaleric, norvaleric and caproic acids were administered as 10% and heptylic acid as 5% aqueous solutions; caprylic, nonanoic, capric, undecylic, lauric, tridecylic and myristic acids as 2% emulsions; pentadecylic, palmitic, margaric and stearic acids as 0.2%, and oleic acid as 2% emulsions.

For investigating the action of fatty acids on *guinea-pig gut*, 1% emulsions of the compositions recorded in table 1 were used.

Cottonseed oil was similarly utilized as carrier when investigating the *respiratory* and *blood-pressure* responses of the cat to higher fatty acids. Emulsification was with phosphatides and Pluronic F 68 (table 2), because they make stabler emulsions of

1) Grass Instrument Co., Quincy, Mass., U.S.A.

2) Statham Instruments Inc., 254 Carpenter Road, Hato Rey, Puerto Rico.

Table 1.

Composition of fatty acid emulsions used for determining the toxicity in mice.

The cottonseed oil, supplied by Wesson Oil and Snow-drift Sales Co., New Orleans, La., U.S.A., was used after filtration at $+4^{\circ}\text{C}$. The phosphatides were prepared as described WRETTLING (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 tridecyllic.

The *cottonseed oil emulsions* used in the cat experiments had the compositions shown in table 2, except that they contained no fatty acid and that either unsaturated phosphatides (WRETTLING 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 $85-130^{\circ}\text{C}$ 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 of acetone and placed in a refrigerator at $+4^{\circ}$. The resulting phosphatides were filtered off under reduced pressure, washed with acetone, and dried in a desiccator under reduced pressure. The yield amounted to 30-50 grams. Analysis showed N 1.6-1.7 per cent and P 3.7-3.8 per cent.

Table 2.

Composition of emulsions used for investigating the effects of fatty acids on respiration and blood pressure.

Pluronic F 68 (polypropylene-polyethylene-glucose) obtained from Wyandotte Chemical Corp., Wyandotte, Ill., U.S.A. The emulsions were adjusted to pH 7.3 with 1 N-NaOH.

Fatty acid	1 g
Cottonseed oil	10 g
Phosphatides	1.2 g
Pluronic	0.4 g
5% glucose solution to	100 ml

Results.

In attempts to determine the LD₅₀ for the carrier emulsions with *cottonseed oil alone*, we found on intravenous injection of a maximal volume - i.e., 60 ml/kg of the 25% cottonseed oil emulsion with emulsifiers, as shown in table 1 - into mice only a transient depression of the respiration rate. It follows that the LD₅₀ is higher than 15 g cottonseed oil per kilogram.

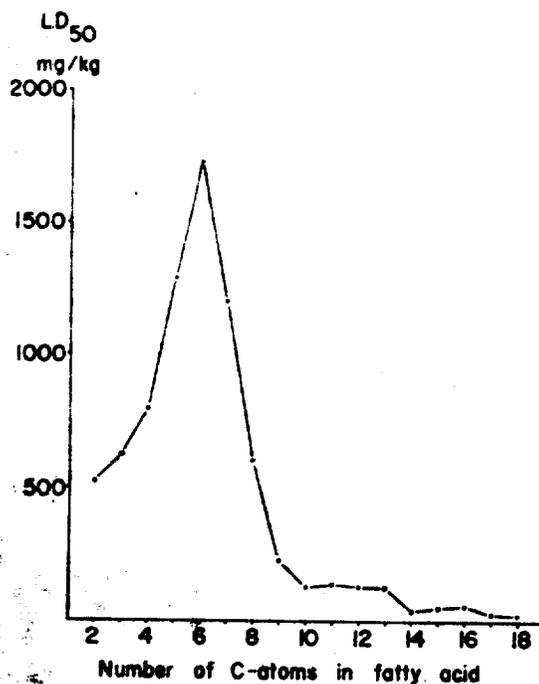


Fig. 1. Toxicity of saturated fatty acids on intravenous injection into mice. The abscissa indicates the number of carbon atoms in the fatty acid molecule; the ordinate, LD₅₀ in milligrams per kilogram of body weight. The LD₅₀ values in mg/kg, together with their standard errors, were: acetic acid 525 ± 21; propionic acid 625 ± 33; butyric acid 800 ± 24; n-valeric acid 1290 ± 53; isovaleric acid 1120 ± 30; caproic acid 1725 ± 85; heptylic acid 1200 ± 56; caprylic acid 600 ± 24; nonanoic acid 224 ± 4.6; capric acid 129 ± 5.4; undecylic acid 140 ± 4.2; lauric acid 131 ± 5.7; tridecylic acid 130 ± 7.0; myristic acid 43 ± 2.6; pentadecylic acid 54 ± 3.2; palmitic acid 57 ± 3.4; margaric acid 36 ± 0.3; stearic acid 23 ± 1.3.

Figure 1 shows that the intravenous toxicities of different fatty acids differ considerably for mice. Least toxic is caproic acid with six carbon atoms, which has an LD₅₀ of 1,725 ± 85 mg per kg body weight. With an increased or decreased number of carbon atoms in the fatty acid molecule, the toxicity rises. The most toxic of all those investigated was

stearic acid, which had an LD50 of 23 ± 0.7 mg per kg. Approximately the same value was obtained for stearic acid in the rat (21.5 ± 1.8 mg/kg). Oleic acid showed an LD50 of 230 ± 18 mg/kg in mice. This implies that stearic acid is ten times more toxic than the unsaturated oleic acid. The toxicity of norvaleric and isovaleric acids differed slightly, LD50 being $1,290 \pm 53$ and $1,120 \pm 30$ 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.

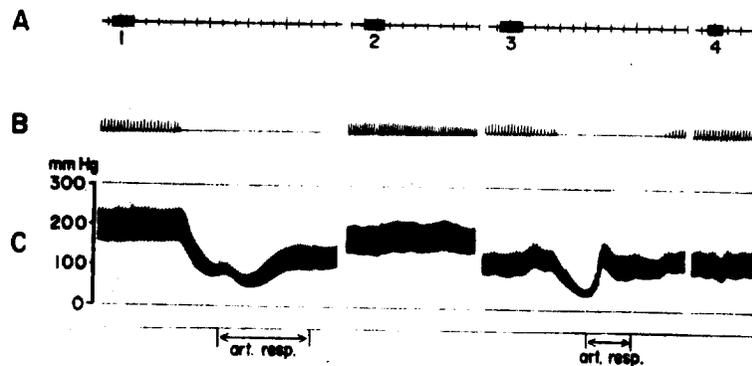


Fig. 2. Influence of cottonseed oil emulsion on blood pressure and respiration. Cat, 3.0 kg, under Dial $\text{\textcircled{B}}$ anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. "Art. resp." denotes artificial respiration. At 1, 2, 3 and 4, 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

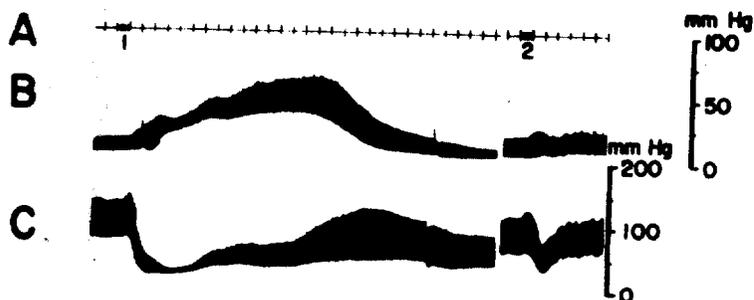


Fig. 3. Effect of cottonseed oil emulsion on pressure in the pulmonary and carotid arteries. Cat 3.3 kg, under chloralose anaesthesia with open thorax. A indicates 10-second intervals and injection times; B, the pulmonary artery; C, the carotid artery pressure. At 1 and 2, 0.5 ml/kg of a 10% cottonseed 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

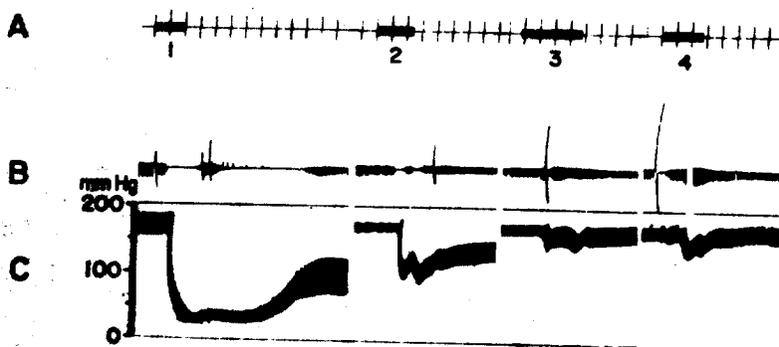


Fig. 4. Effect of phosphatides on blood pressure and respiration. Cat, 3.8 kg, under Dial @ anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, the blood pressure in mm Hg. At 1, 2, 3 and 4 4 ml/kg of 2.4% solution of soya bean phosphatides and 0.2% Pluronic F 68 were injected. The phosphatides have a hypotensive action characterized by tachyphylaxis, though a slight effect persists.

the cottonseed oil, but on repeated injection these quickly subsided because of the tachyphylaxis. With fatty acid emulsions containing hydrogenated phosphatides, only the effects of the fatty acids were observed.

Intravenous injection of the *fatty acids* investigated – stearic, oleic, lauric and tridecylic – had a hypotensive effect on the systemic *circulation*, but raised the pulmonary arterial pressure. The doses required for this action were 5 mg stearic acid, 50 mg oleic acid, 75 mg lauric acid or 60 mg tridecylic acid per kg body weight. All of the fatty acids studied had a negatively inotropic effect on the heart, as shown by the experiments on heart-lung preparations. Similar results were given by oleic acid emulsions

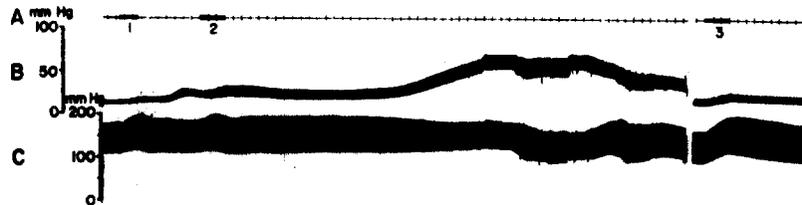


Fig. 5. Influence of triolein emulsion on blood pressure and pulmonary arterial pressure after vagotomy. Cat, 4.0 kg, under Dial [®] anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, the pressure in the pulmonary artery; C, the arterial blood pressure in mm Hg. Four ml/kg of 10% triolein emulsion with pluronic F 68 only as emulsifier were injected intravenously at 1, and 7 ml/kg at 2 and 3. It will be seen that the emulsion caused a rise of blood pressure in the pulmonary artery with a slight secondary fall in blood pressure, effects that disappeared on repeated injection.

without cottonseed oil as carrier and also by those with unsaturated phosphatides and Pluronic F 68 as emulsifiers. Emulsions with different emulsifiers differed somewhat in their effects. For instance, the effect on pulmonary arterial pressure was far less with hydrogenated than with unsaturated phosphatides. The action on the *respiration* was characterized by hyperpnoea after small doses and apnoea after larger ones. No tachyphylaxis was observed with fatty acid emulsions. In several experiments, indeed, an increased effect was noted on repeated injection of equal doses (figures 6 and 7). – Vagotomy had no effect on falls in blood-pressure produced by the fatty acids studied.

As to the action of *fatty acid emulsions on small intestine from the guinea pig*, our results indicated that the emulsions did not themselves cause any contraction, even in relatively large doses (100 mg/15 ml). They could, however, inhibit the contraction produced by histamine. The degree of inhibition depended on the particular fatty acid contained in the emulsion. Fig. 8 shows the relative potencies of the fatty acids in this respect. It will be seen from the curve that capric and undecylic acids, i.e., those with 10 or 11 carbon atoms, were the most effective. Somewhat more than 15 mg/15 ml of these acids was required for total inhibition of

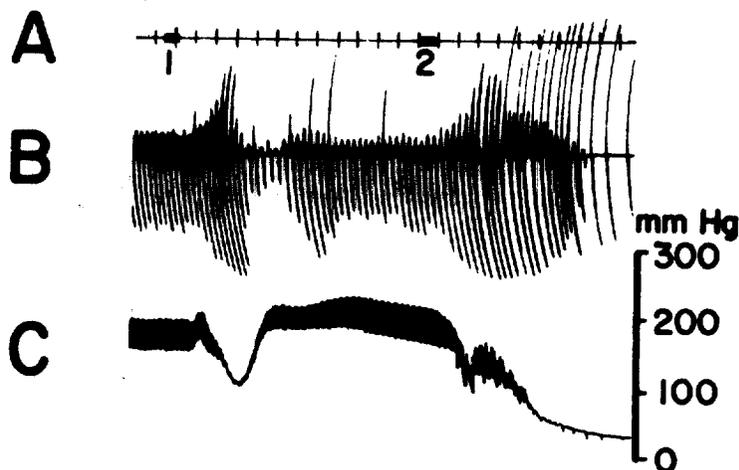


Fig. 6. Effect of oleic acid emulsion on blood pressure and respiration. Cat, 4.0 kg, under chloralose anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. One ml/kg of 10% oleic acid emulsion in cottonseed oil (10%) with soyabean phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at 1 and 2.

the contraction after 0.2 μ g histamine. If the fatty acid molecule contained a greater or smaller number of carbon atoms than 10 or 11, the relaxing action on histamine-contracted intestine decreased; for instance, 150 mg of butyric acid was required for total inhibition. Comparison of undecylic acid with its unsaturated homologue, undecylenic acid, revealed no significant difference. The emulsion system alone without fatty acid is inert. No difference was detectable when the lower fatty acids were administered in aqueous solutions instead of in emulsions.

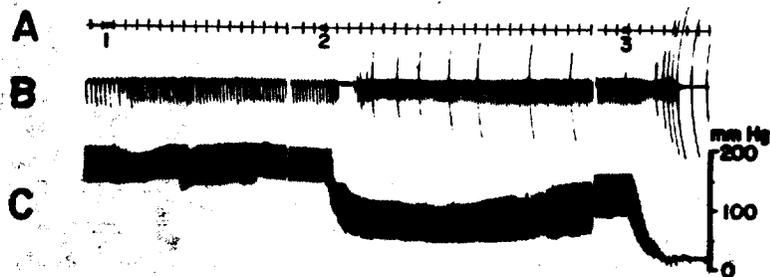


Fig. 7. Effect of stearic acid emulsion on respiration and blood pressure. Cat, 4.3 kg, under chloralose anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. At 1, 2 and 3, a 1% stearic acid emulsion with 10% cottonseed oil as carrier and phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at a dose of 0.2 ml/kg.

FATTY ACIDS

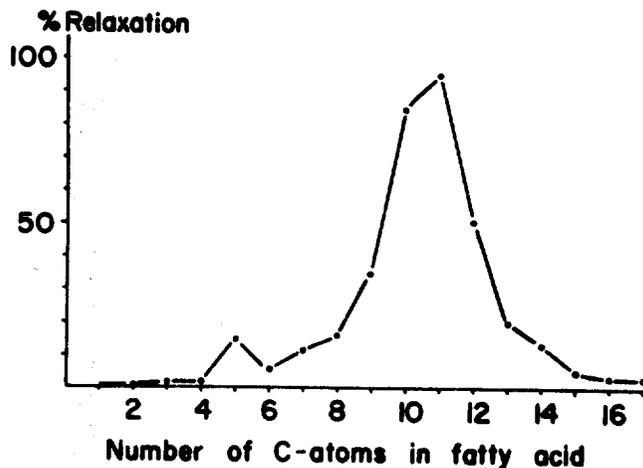


Fig. 8. Relaxing action of fatty acids on guinea-pig small intestine contracted by histamine. The fatty acids were administered in the form of emulsion. Abscissa indicates the number of carbon atoms in the fatty acid molecule. Ordinate shows percentage relaxation of guinea-pig intestine contracted by 0.2 μ g 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 (C₂-C₁₀). There seems to be no direct correlation between hypnotic and toxic effects. The ED50 for hypnotic action decreases progressively with an increasing number of carbon atoms in the fatty acid molecule, in contrast to the LD50, the highest dose being that of caproic acid with six carbon atoms and the dose then falling with both increased and reduced numbers of carbon atoms.

The responses of respiration and blood pressure to the fatty acid emulsions differed from those to the triglycerides of low molecular weight

(WRETLIND 1957 a); in cats intravenous injection of more than 5 mg stearic acid or 50 mg oleic acid per kg was followed by apnoea and a fall in blood pressure as well as by convulsions with lethal outcome. Lower doses had a depressor action on blood pressure; the respiration sometimes being stimulated. The dose required for toxic action varied somewhat with the emulsifier employed. The effect on the circulatory system also differed with different emulsifiers. Stearic acid emulsions with phosphatides and Pluronic as emulsifiers raised the pulmonary arterial pressure substantially, and this elevation contributed to the fall of blood pressure in the systemic circulation. With hydrogenated phosphatides there was no demonstrable influence on pulmonary arterial pressure; on the other hand these emulsions had a pronounced effect on heart-lung preparations, so that their hypotensive action may be attributable to direct cardiac depression. Oleic acid emulsions that had been emulsified solely with phosphatides and Pluronic F68, without cottonseed oil, also had a hypotensive and toxic action on heart-lung preparations.

It is accordingly evident that the emulsified higher fatty acids studied had, on intravenous injection, a pronounced toxic action associated with a fall of blood pressure.

PELTIER (1956) determined the toxicity of some neutral fats and free fatty acids in non-emulsified form. He concluded that the toxic effects of fatty acid were caused by damage to the pulmonary capillaries, with extravasation into the alveolar spaces. JEFFERSON (1948) held a similar view. In our experiments on anaesthetized cats we occasionally observed progressive respiratory distress, and blood-tinged froth escaped from the tracheal cannula. At autopsy the lungs were oedematous. - It is worthy of note that the LD50 is more or less the same for non-emulsified oleic acid in rabbits (PELTIER 1956) and emulsified oleic acid in mice (250 mg/kg and 230/kg respectively). SCUDERI (1941) found that 0.33 ml of non-emulsified oleic acid per kg had a toxic action on the dog. The emulsified form had a lethal effect on anaesthetized cats in somewhat smaller doses, i.e., 50-100 mg/kg. However, total amounts substantially greater than 250 mg of emulsified oleic acid per kg can be given by repeatedly injecting sublethal doses.

The fatty acid emulsions had no stimulating action on *small intestine from the guinea pig* - a finding that accorded with results reported by GABR (1956). The emulsions could, however, relax histamine-contracted intestine. This effect was not correlated with their toxic action; rather the reverse, the relaxing effect being maximal for fatty acids with 10 or 11 carbon atoms and decreasing with either a smaller or greater number of carbon atoms in the molecule. Of interest in this connection is the finding of GANLEY *et al.* (1960) that higher fatty acids partially inhibited the

action of serotonin (5-hydroxytryptamine) and the effect in anaphylactic shock in mice.

As to the blood pressure fall that follows injection of emulsions of *cottonseed oil* or *triolein*, it is difficult to determine the mechanism exactly because of the pronounced tachyphylaxis.

If an anaesthetized cat is injected with 6 ml/kg 10% emulsion of cottonseed oil or triolein emulsified with 0.1 or 1.0% Pluronic F 68, a substantial fall in blood pressure as well as apnoea occur after about 10-60 seconds. The cat may even die after this first injection. The pulmonary arterial pressure sometimes rises as high as 100 mm Hg. Our results suggest that the hypotensive effect is elicited mainly via the vagus, for it is practically eliminated by vagotomy (fig. 5).

Since the responses of blood pressure can also be obtained with the soya bean phosphatides alone, it seems probable that the effects observed with the cottonseed oil and triolein emulsions were due to the presence of such phosphatides. That the unsaturated phosphatides were responsible also appears likely, inasmuch as there was no circulatory response when hydrogenated soya bean phosphatides were used as emulsifiers. The fact that cottonseed oil emulsions with Pluronic F 68 as the sole emulsifier produced similar effects on the blood pressure shows that this is not the only explanation. Our observations tend rather to suggest that the effects of intravenously administered fat emulsions are due to a number of factors, such as the properties of the fat, the emulsifiers, the surface tension, size of fat particles and the charge carried by them. On the basis of our present knowledge in this field, it is impossible to say finally which is the principal factor involved.

According to SEEVERS (1958), tachyphylaxis is probably due to specific "receptors" that may take up the active substance, thereby eliciting the pharmacological action. When no more of these receptors are available, the effect ceases. The active substance is subsequently released slowly, and not until the liberation is complete can an effect be once more obtained. In our experiments the recovery period was relatively long, at least four hours. It is not yet possible, however, to say where these receptors are situated or by what mechanism the fat emulsions block them.

Summary.

Pharmacological investigations of the effects of intravenously administered fatty acids of high molecular weight are handicapped by the fact that such acids are insoluble in water at neutral reaction. By utilizing fat particles in an emulsion as carriers of these fatty acids, it has been possible

in our investigation to study the toxicity and certain pharmacological effects of such acids when given intravenously. Although emulsions of the carrier fat are devoid of toxicity, they exhibit peculiar pharmacological effects characterized by pronounced tachyphylaxis. In this investigation the following observations were made.

The LD50 by intravenous injection into mice was determined for saturated *fatty acids* from C₂ to C₁₈. 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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Gastric Cancer: Possible Dietary Carcinogens

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Probably at least half the deaths from cancer in the white population of the world are due to tumours of the alimentary canal, and more than half of these are cases of cancer of the stomach. In spite of educational and propaganda campaigns and improvements in surgical technique, the prospect of survival for the average patient with gastric cancer has not been very greatly improved, and most patients correctly diagnosed as having this disease die of it within a few years.

Naturally a great deal of research has been devoted to this subject and as it has been admirably reviewed by Klein and Palmer (1), and more recently by Barrett (2), only a few contributions will be mentioned in this communication.

Demographical and geographical studies of human pathology suggest very strongly that environmental factors are of more aetiological importance than are hereditary or ethnic characters in cancer of the exposed sites, including the alimentary canal as far as the pylorus. In this connection Kennaway (3) has recently drawn attention to the valuable information available in the Registrar General's Decennial Supplements, and in particular that for 1921 (4) which "contains the first detailed study of the social incidence of cancer upon the various organs in man, which study developed from an earlier investigation of the incidence of cancer as a whole" (Stevenson, 1923) (5).

Stevenson analysed the Registrar General's returns for England and Wales, dividing the population into five sociological groups: the upper two groups comprised those most comfortably off, the professional and leisured classes; and the lower two groups the worst off. The middle group accounted for the great majority of the middle-class population. In these five groups the incidence of cancer of the exposed sites, including the stomach, was more than twice as high in the fifth group as it was in the first two. That these results were genuine and were not merely due to the greater proneness of the poor population to be examined *post mortem* in hospitals, was shown by the almost equal incidence of cancer of the rectum in all five sociological groups.

In the Decennial Report for England and Wales for 1931 (6), the figures for the same five groups were similar, but the differences were less striking, reflecting the levelling of social groups during the period under review. Recently Clemmensen (7) has made careful studies of the fairly comparable statistics for Denmark, Switzerland, and England and Wales, and has shown that the differences in cancer mortality between these three countries, at least for the male population, are mainly due to the greater incidence of gastric cancer in Switzerland and Denmark as compared with England and Wales.

That such differences are more likely to be due to environmental factors than to hereditary or ethnic factors is suggested by the geographical distribution of cancer of the alimentary canal within an ethnically homogeneous population, as in the material studied by Stevenson. Stocks (8) has shown that in the large towns of England and Wales there is a lower incidence of gastric cancer in towns with a moderately hard water supply than in those with either very soft or very hard water, and that such

¹ Now re-named the Royal Beatson Memorial Hospital.

differences are not likely to be due to chance. However, there are a great many other factors that might be equally or more important in determining tumour incidence and it is hard to show that coincidental data are causally related in such a study. Legon (9) has recently recorded that there is some correspondence between peaty soils and the incidence of gastric cancer in England and Wales; but again it has not been shown that this is of aetiological importance.

The establishment of a group to study the demographical and geographical pathology of cancer by the International Cancer Research Commission of the Union Internationale Contre le Cancer may be expected to yield useful results in correlating work of this kind in different parts of the world.

Animal statistics, though much less reliable than human, show that gastric cancer is one of the rarest forms of malignant disease in all the species that have been studied. It may be objected that the majority of animals examined *post mortem* are killed at a relatively early age and that if the great majority of human beings were similarly killed in the early prime of life, there would be few cases of gastric cancer. Yet there are a sufficient number of animals kept to old age, in the case of pets or in zoological parks, for certain figures to be of value. Thus a special number of the *Onderstepoorte Journal of Veterinary Science* was devoted to the consideration of tumours in elderly animals in the National Zoological Parks in South Africa. In this study Jackson (10) described a single case of gastric adenocarcinoma in a horse, amongst 119 malignant tumours in the species. Cancer in other domestic animals was not uncommon in his experience, but he reported no other cases of gastric cancer in domestic or wild animals.

The earliest claim to have induced gastric cancer, at least in the forestomach of rats, was made by Fibiger (11) who had studied what he considered to be spontaneous cancer in wild rats infested with a round worm, the *Gongylonema neoplasticum*, whose intermediate host is the American cockroach, *Periplaneta americana*. Fibiger fed rats experimentally with this parasitic worm and observed the development of enormous papillomatous masses in the forestomach, some of which he considered to be malignant.

Some of Fibiger's illustrations seem to the author to support a diagnosis of at least local squamous-cell carcinoma in the forestomach, but subsequent workers have failed to reproduce his results. Many of the lesions that he observed have been attributed by Passey, Leese, and Knox (12, 13) to squamous metaplasia in bronchiectatic cavities, which could easily be mistaken for secondary deposits of squamous-cell carcinoma, these lesions being associated with a deficiency of vitamin A which of course could not have been recognized in 1913, at the time of Fibiger's experiments. In any case, such experiments probably have little bearing on the occurrence of gastric cancer in the human subject. The striking difference between the incidence in humans and in animals of this type of cancer has led many investigators to suspect that some essentially human habit, connected with food for example, and in particular with the consumption of overheated foods, may be of great aetiological importance.

Our interest in heated fats began in experiments in no way connected directly with food, with the observation by one of us [P.R.P. (14)] in 1933 that a sample of lard used as a solvent for 1:2:5:6-dibenzanthracene and injected into fowls caused sarcomata to grow not only at the site of injection of the solution of carcinogen (in 10 out of 31 birds), but also at the control site of lard injection in the opposite breast muscle in 3 out of the same 10 birds. Three years later Kennaway and his colleagues (15) at the London Cancer Hospital described apparently nonmalignant fibromatous tumours at the site of injection of lard and other fats used as solvents for carcinogens. Subsequent work in our own and other laboratories has confirmed the fact that heated fats can sometimes act as carcinogens in the connective tissues of rats and mice.

Relfo (16) claimed to have induced three kinds of tumours in rats maintained on a diet consisting of 50 percent bread and milk and 50 percent heated fats. Beef fat, mutton fat, pig fat, and olive oil were heated to temperatures of about 300° C. for half an hour. Rats fed on such fats were said to develop squamous papillomas or carcinoma of the forestomach, adenocarcinoma of the glandular stomach, and spindle-

cell sarcoma of the liver. However, subsequent workers have failed to substantiate any but the first of these types of tumours; and Roffo's own illustrations of what he called adenocarcinoma of the glandular stomach do not carry conviction. Moreover, the sarcomata of the liver were very similar to those commonly seen in rats infested with *Cysticercus fasciolaris*, and as Roffo does not seem to discuss this agent as a possible factor in his experiments, one cannot accept such tumours as evidence of the carcinogenic potency of the heated fat in the diet.

In our attempts to repeat Roffo's experiments we observed (17) some small papillomata of the forestomach in 2 out of 4 mice, 12 months old, that had been fed for 9 months with a mixed cooking fat heated in an iron saucepan to between 200° C. and 220° C. for 12 hours; but we found only lesions of the kind associated with avitaminosis A in rats similarly fed. That is to say, extensive papillomatosis and ulceration occurred, but such lesions could be prevented or cured by the addition of raw carrot to the diet, and there was no tendency to malignant growth in the forestomach.

Morris, Larsen and Lippincott (18) repeated Roffo's experiments with lard heated to 350° C. for 30 minutes. They found no tumours in the glandular part of the stomach. "Gross examination of the forestomach usually disclosed no apparent lesions. However, in 9 of the animals this organ presented varying numbers of discrete, white, nodular elevations of the mucosa measuring up to 3 × 2 mm. Microscopically such elevations proved to be benign papillomas. . . ." Apart from these, ulcers were sometimes found. They also noticed gross damage to the liver in their experimental animals, some of which showed cirrhotic as well as fatty lesions. Only 2 of their rats given the fat heated to 350° C. seem to have survived for as long as 75 weeks, and this may account for their failure to observe more advanced lesions in the forestomach.

Ivy and his group (19) at Chicago have also repeated Roffo's experiments on a considerable scale, using rats of the same strain as employed by Roffo, and have induced papilloma or hyperplasia of the marginal ridge, ulceration or petechial haemorrhages in the glandular part of the stomach, in 37 percent of the rats fed a diet of bread and milk and lard heated to 350° C. for 30 minutes in an open kettle, as compared with 5.7 percent in controls with unheated lard. They also obtained some tumours, including a sarcoma, amongst rats injected subcutaneously with such heated lard, but there were no malignant tumours in their feeding experiments.

To the best of our knowledge no one has induced adenocarcinoma in the glandular stomach, such as occurs in man, in any other species by any experimental diet. However it has been shown, first by Stewart and Lorenz (20) for mice and rats, and later confirmed by Howes and Oliveira (21) for rats, that the rodent's glandular stomach is capable of growing adenocarcinoma after methylcholanthrene has been introduced into the gastric wall. Even such drastic procedures as the induction of ulcers by bilateral vagotomy and excision of mucosa, by Denton, Sheldon and Ivy (22), have failed to render the glandular stomachs of rodents susceptible to the action of prolonged exposure to methylcholanthrene.

Lushbaugh (23) in some interesting experiments begun for another reason with aerosols of lubricant oil, found many lesions in the stomachs of Rhesus monkeys exposed to the vapours of these oils for prolonged periods, and yet in spite of the suggestive appearances, none developed undoubtedly neoplastic lesions. Pfeiffer and Allen (24), also using Rhesus monkeys, failed even after the most prolonged and drastic exposure to quantities of up to 6.5 gm. of methylcholanthrene over a period of up to 10 years, to induce any malignant lesions at all.

There can be no doubt that research into gastric cancer is greatly hampered by the lack of any convenient small, short-lived mammal with a purely glandular stomach. The rodents that are so extensively used in laboratories have a squamous-lined forestomach or rumen which has no counterpart in the human and which is particularly liable to develop papilloma or squamous-cell carcinoma in response to the action of the known carcinogenic hydrocarbons. In investigating a problem that affects heterozygous human beings we find it logical to use heterozygous mice. Our mouse

colony has been bred within the laboratory without introduction of new stock for many years and might be compared with the population of an isolated country.

In planning our experiments with heated fats added to the diet, we have tried to keep the experiments within conditions that are not too severe on the mice. It is perhaps worth remembering that cancer of the stomach in the human, whatever its cause, occurs in middle or late life and in people who have not complained, as a rule, of any very severe digestive troubles in their earlier years. It therefore seems foolish to maintain animals on diets that render them acutely miserable early in the experiment. Strongly heated fats taste and smell unpleasant, and animals will avoid eating food contaminated with them in a high concentration. After trying various techniques, we found that one way of administering fats was as a 1 percent suspension in a 10 percent soap solution, which curiously enough mice do not seem to dislike. Such a solution was given in drinking bottles to several groups of mice. We thought that by this procedure we might bring the presumed carcinogen in the heated fat into intimate contact with the mucosa of the glandular part of the stomach. However, as soap solution is not a human dietary factor we have used, in recent experiments, heated cottonseed oil added to dry rat-cake in a mechanical mixer to yield a 5 percent mixture by weight.

With a view to testing possible co-carcinogenic actions, we have added a variety of substances to such aqueous soap suspensions of heated fats: croton oil in concentrations of 0.05 percent and 0.1 percent; black pepper to the extent of 0.5 percent mixed with the dried food; and "Icrol" sulphate, a mixture of sodium dodecyl and tetradecyl sulphates, have been added in certain experiments (table 1).

The earliest papilloma of the forestomach observed in our experiments occurred in a mouse 407 days old, and we therefore consider only the tumour incidence in mice aged more than 400 days and fed on the experimental or control diet for about 300 days. In other experiments we substituted 10 percent or 40 percent ethyl alcohol for soap solution as a solvent for 1 percent heated cottonseed oil. The results of our experiments are shown in table 1. In a total of 268 mice aged over 400 days and having ingested heated cottonseed oil in one form or another daily throughout most of their lives, 18 developed papillomata, 6 carcinoma of the forestomach, 7 showed some degree of adenosis of the pyloric stomach, and 15 developed hepatomata; 4 had adenomata and 1 an adenocarcinoma of the lung; 1 had a sarcoma of the right leg; 1 a lymphosarcoma and 1 a reticulosarcoma of the liver; 5 females had mammary tumours. The remote tumours, however, except the hepatomata, were not considered to be in excess of those expected in control groups of animals. Thus about 9 percent of the middle-aged and elderly mice on the heated cottonseed-oil diet had tumours of the forestomach, and about 5 to 6 percent had hepatomata.

Representative tumours are illustrated in figures 1, 3 to 8, and 10.

Amongst the various controls, which included all the solvents used, without the heated cottonseed oil or with the addition of unheated cottonseed oil, there were 99 survivors amongst which 3 developed papillomata of the forestomach; 3 adenosis of the glandular part of the stomach; and 1 hepatoma. Among 86 untreated controls maintained on the same basal diet without any additions, there were no tumours of the stomach but 1 hepatoma. Thus in 185 controls there were 3 papillomata of the forestomach, 3 cases of adenosis, and 2 hepatomata (table 2), an incidence of about 2 percent forestomach tumours and 1 percent hepatomata.

Though these numbers are small, it seems to us that they may have some significance. In every experiment malignant tumours have occurred only in the group with the heated cottonseed oil as compared with the corresponding control group, but soap solution may have been instrumental in inducing papillomata in both groups. However, heated cottonseed oil alone has induced 6 papillomata and 2 carcinomata among 63 mice (table 1).

During the same period in the laboratory we have, however, seen 3 cases of cancer of the stomach in mice of the same stock in experiments which were not expected to yield such lesions. One of these was injected intraperitoneally with ortho-amino-

TABLE I.—*Tumour incidence in mice fed heated cottonseed oil (and possible co-carcinogens) surviving over 400 days*

Cottonseed oil heated at:	Percent	Solvent	Plus	No. of mice	Papilloma	Carcinoma	Adenosis	Hepatoma
350° C. × 4 hr.*	5		Histamine (0.5 mg. subcutaneously 3 × weekly).	12	0	0	1	2
" "	5			31	3	0	0	0
320° C. × 2½ hr. + Fe*	5			32	3	2	1	2
350° C. × 4 hr.	1	10% soap solution.		56	3	1	0	4
" "	1	"	0.1% croton oil.	21	1	1	2	0
" "	1	"	0.05% croton oil.	35	4	0	2	2
" "	1	"	2% "lorol" sulphate†.	18	2	1	0	2
" "	1	"	0.5% pepper.	37	2	1	0	2
" "	1	10% C ₂ H ₅ OH.		9	0	0	0	0
" "	1	"	0.05% croton oil.	9	0	0	1	1
" "	1	40% C ₂ H ₅ OH.		8	0	0	0	0
Total				268	18	6	7	15

*Heated in an atmosphere of nitrogen.

†"Lorol" sulphate is a mixture of sodium dodecyl and tetradecyl sulphates.

TABLE 2.—*Tumour incidence in control mice fed unheated cottonseed oil, or no cottonseed oil, and surviving over 400 days*

Percent unheated cottonseed oil	Solvent	Plus	Number of mice	Papillomas	Carcinoma	Adenocarcinoma	Hepatoma
1	10% soap solution		22			2	
0	"	0.05% croton oil	10				
0	"	"	15	1			
0	"	"	24	2			1
1	10% C ₂ H ₅ OH		8				
0	40% "		8				
0	"	Histamine (0.5 mg. subcutaneously 3 X weekly)	12			1	
0	"	"	86				1
	Control total		185	3	0	3	2

phthalic cyclic hydrazide and at death the peritoneal cavity was found to contain widely dispersed remnants of the injected material. This substance is now being tested for possible carcinogenicity. The second mouse was injected subcutaneously with a sample of fat that had actually been used in cooking, but had no lesion at the site of injection (fig. 9). The third anomalous case was in an animal that had been painted once only with 9:10-dimethyl-1:2-benzanthracene and which must undoubtedly have swallowed some of this potent carcinogen. It is impossible to say whether these stomach tumours were related to these experimental procedures, but further tests may establish or refute this possibility.

In an attempt to overcome the drawback of the squamous forestomach in rodents, already referred to, rumenectomy (removal of the forestomach) was practiced by one of us (P. R. P.) on 78 hamsters. This animal has an almost separate rumen with a constriction separating it from the glandular part of the stomach, which makes removal relatively easy. Moreover, animals so treated show no signs of ill-health. With a view to inducing a condition of hypochlorhydria we added hexamethonium bromide to the diet of such rumenectomized hamsters along with heated cottonseed oil in a mixture consisting of:

1,000 gm. oatmeal
20 ml. heated cottonseed oil (350° C. X 4 hrs.)
15 ml. "Beplex"
0.1 ml. cod liver oil
0.075 gm. hexamethonium bromide

Among 41 survivors aged more than 300 days, 1 had a large branching papilloma arising from the small stump of the rumen that had not been removed (fig. 7), and another showed what might have been early stages of a similar lesion. Further experiments are being carried out to ascertain a) whether the operation of rumenectomy alone predisposes the animal to papilloma development at the scar, and b) whether intact hamsters on the heated cottonseed-oil diet develop stomach tumours.¹

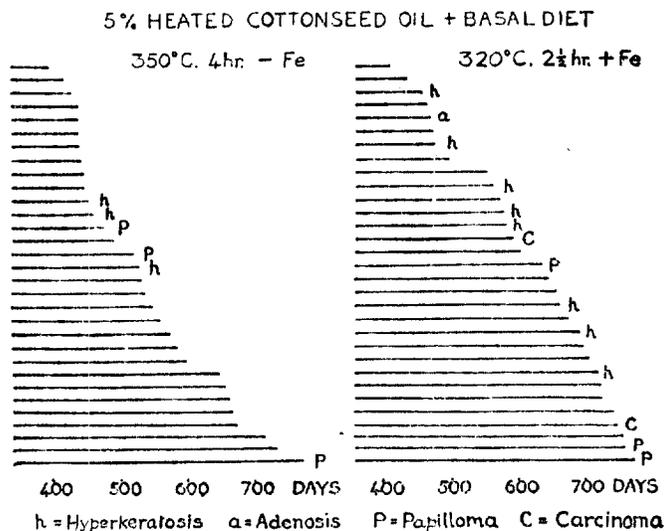
¹ Since this paper was presented I have injected hamsters on the same diet but was killed on the 47th day and found at least 3 separate sessile papillomas at the rumen.

Discussion

In our original experiments with heated lard, we used an open iron pot and noticed that the lard became very black on heating, but that it was much less so when heated in glass vessels. At the time we attached no particular significance to this fact, but on looking into the matter we found that iron is known to catalyse the pyrolysis of fats and the chemical constitution of the resulting products is quantitatively different when the fat is heated in contact with iron. This may be a point of more than academic interest in view of the widespread and time-honoured use of iron cooking vessels, particularly the open frying-pan in which temperatures of at least 327° C. are readily reached, as can be shown by putting small pellets of lead into the pan during cooking, when they will often be seen to melt.

It was suggested by Morris, Larsen and Lippincott that ketonisation occurred in heated fats, and this has been the case in our experiments. Other changes include polymerisation, though the exact nature of the compounds formed has not been established. In the laboratory it was found impracticable to heat cottonseed oil to 350° C. in the presence of iron filings because of the vigorous reaction that occurs, and the temperature employed when iron was added was 320° to 325° C. and the time 2½ hours.

A comparison between such heated cottonseed oil and a similar sample heated to 350° C. for 4 hours without contact with iron was made, and is included in table 1 and separately treated in text-figure 1. The group of mice treated with the iron-catalysed heated cottonseed oil show a higher proportion of gastric lesions than those with the more strongly heated oil in the absence of iron. However, the former group had a longer average survival. Table 3 shows some of the differences in the end products when cottonseed oil is heated under these different conditions.



TEXT-FIGURE 1.—Comparison between cottonseed oil heated with and without iron filings as catalyst.*

* Of the mice given cottonseed oil with iron filings, 2 mice died on the 468th day. Through an error, only one line for this day was recorded (second column, 7th line down).

TABLE 3.—List of heat transformation products of cottonseed oil

Product	300°/28 hrs. (percent)	350°/4-6 hrs. (percent)	320°/2-3 hrs. with iron filings (percent)
Glycerides (isomerised and polymerised)	55-60		
Free fatty acid	30	30-40	5-10
Non-saponifiable material (ketones)	20-30	30-40
Sparingly soluble material (tar)	10-50	50

From the chemical point of view, there is no doubt that decarboxylation occurs at a lower temperature in the presence of iron. This can be shown by passing a stream of nitrogen over the heated oil and noting at what temperatures carbon dioxide is given off. When cottonseed oil is heated without iron under these conditions, CO₂ is detected first at 345° C.; when iron filings are added it is detected at 250° C.; and when iron strips are added, at 305° C. Polymerisation undoubtedly occurs, as indicated by an increase in viscosity of the solution after heating for various lengths of time, and this increase of viscosity is accompanied by the production of free fatty acid from the oil.

In the experiments marked with an asterisk in table 1 the cottonseed oil was heated in an atmosphere of nitrogen, and it may be objected that this is not comparable to cooking conditions. However, when heated in a deep vessel, fat rapidly displaces the air with oil vapour and the purpose of the nitrogen atmosphere was simply to prevent combustion, which is liable to occur in open vessels at temperatures approaching 350° C. Tumours have been induced with cottonseed oil heated with and without the nitrogen atmosphere.

Whatever may be the carcinogen in heated cottonseed oil, and in other fats heated under similar conditions, it is not associated with a banded fluorescence spectrum and it is almost certainly not 3:4-Benzpyrene, because repeated spectrographic tests for that substance have failed to reveal it by methods that will detect one part in ten million. Other workers seem to be in agreement with this (19).

Recently a very interesting suggestion has come from the Imperial Chemical Industries group of workers at Manchester. Hendry, Homer, Rose, and Walpole (25) have described in 1951 a considerable number of new synthetic substances in three distinct groups, each of which has been shown to have cytotoxic, mutagenic, and in some cases carcinogenic properties. They suggested that our heated cottonseed oil might contain organic epoxides of a type that they have shown to be carcinogenic, and that glycidyl stearate or some related compound might be one of the substances responsible for the carcinogenicity of heated cottonseed oil (25). If such a reaction takes place, the glycidyl compounds formed from cottonseed oil would contain chiefly the palmitate; at the temperature of 350° C. this substance would probably be decomposed.

Among the substances described by the Imperial Chemical Industries group are a number of nitrogen-containing substances which, though they could not be formed from heated cottonseed oil alone, might conceivably be formed when protein foods are cooked in fats at high temperatures. So far we have not been able to identify in our heated cottonseed oil any of the substances described by Hendry and his colleagues; but the possibility of their presence has been by no means excluded.

Another type of chemical change that might occur in heated fats is the migration of acyl groups that has recently been shown to take place when triglycerides are heated in glass vessels at about 300° C., as shown by Barker, Crawford and Hilditch (26). In the case of oleodistearin, equilibrium was reached in 10 hours as follows: tristearin 25 percent; oleodistearin 54.2 percent; steardiolein 20.2 percent; oleoilein 0.3 percent. The only side reaction detected was a slow production of free fatty acids.

The almost complete absence in animals, not only of adenocarcinoma of the stomach, but of gastric ulcer and of pernicious anaemia, may perhaps give us a clue to the necessary pre-cancerous lesions that favour the development of cancer of the human stomach. In this connection recent work described by Roca de Vinals (27) may have some importance. He showed that by repeated histamine injections into mice, hyperplastic lesions could be developed not only in the forestomach but also in the glandular stomach. We have repeated his experiments and can confirm that hyperplastic lesions, though not so striking as those described by him, occur in the glandular stomach as well as in the forestomach in our mice. We have combined a heated cottonseed-oil diet with thrice weekly subcutaneous injections of 0.5 mg. of histamine, and have observed in mice so treated very considerable damage and degenerative changes in the glandular part of the stomach, resulting after about 600 days in a condition resembling atrophic gastritis. In some of these stomachs peculiar anomalous glandular proliferations seem to take place, but we have not seen any unquestionable neoplastic changes. Nor did Roca de Vinals claim that any such lesions occurred in his mice.

While the results of our experiments are somewhat disappointing in that no adenocarcinoma of the glandular stomach has been induced, and although the procedures adopted are still in many ways very artificial as compared with human habits, nevertheless they seem to offer possibilities for considerable further development and we believe that they are worth pursuing.

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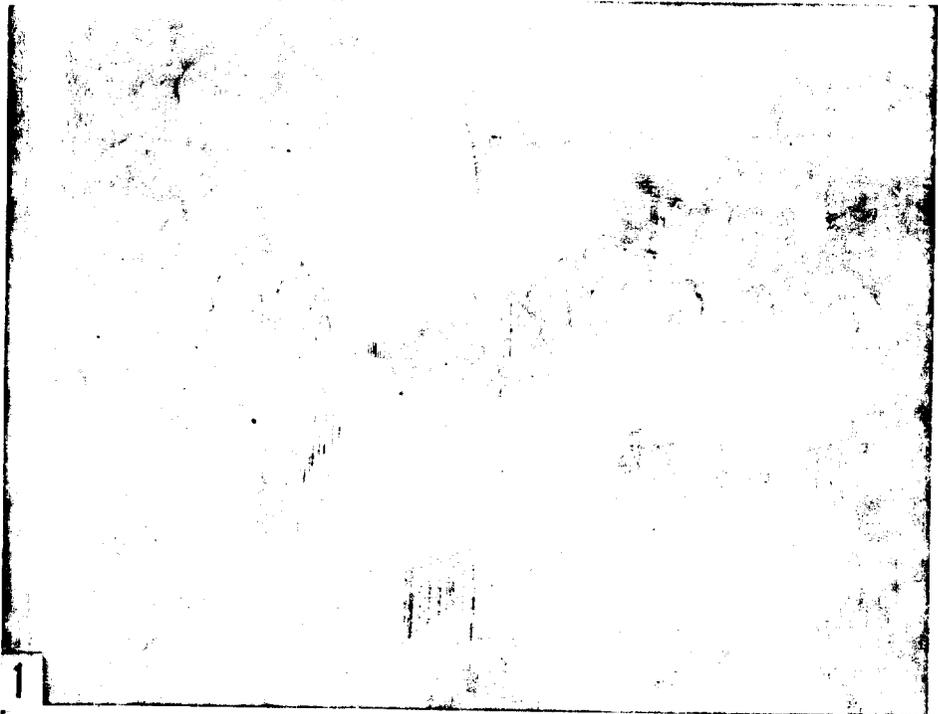
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 PLATE 99

FIGURE 1.—Mouse No. 466/49, age 844 days. The fundus of the stomach is free from tumour. The remainder of the stomach is extensively invaded by squamous-cell carcinoma and the lumen is encroached upon by tumour, part of which is still clearly papillomatous. The tumour extends through the pylorus and projects into the duodenum. There were metastases in peritoneum, lungs, and kidney. $\times 3$

FIGURE 2.—Hamster (male) No. 313/51, age 833 days. Partial rumenectomy had been performed 740 days previously. Diet: crushed oats, heated cottonseed oil (350° C. $\times 4$ hours), croton oil, "Beplex," hexamethonium bromide, plus fresh greens as available and kitchen scraps. A sessile fleshy papilloma occupies the site of the operation scar. No histological evidence of malignancy. Glandular stomach normal. $\times 2$



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FIGURE 3.—Mouse No. 466/49, age 844 days. Hemi-section of stomach (left half of fig. 1). The tumour tends to form intracystic papillary growths. The fundus is lined by septic granulations. A small portion of the glandular mucosa is normal; the remainder is replaced by squamous-cell carcinoma. Haematoxylin and eosin. $\times 4$

FIGURE 4.—Mouse No. 466/49 (same as figs. 1 and 3), age 844 days. Shows squamous character of the carcinoma and the usual tendency to central degeneration. Haematoxylin and eosin. $\times 480$

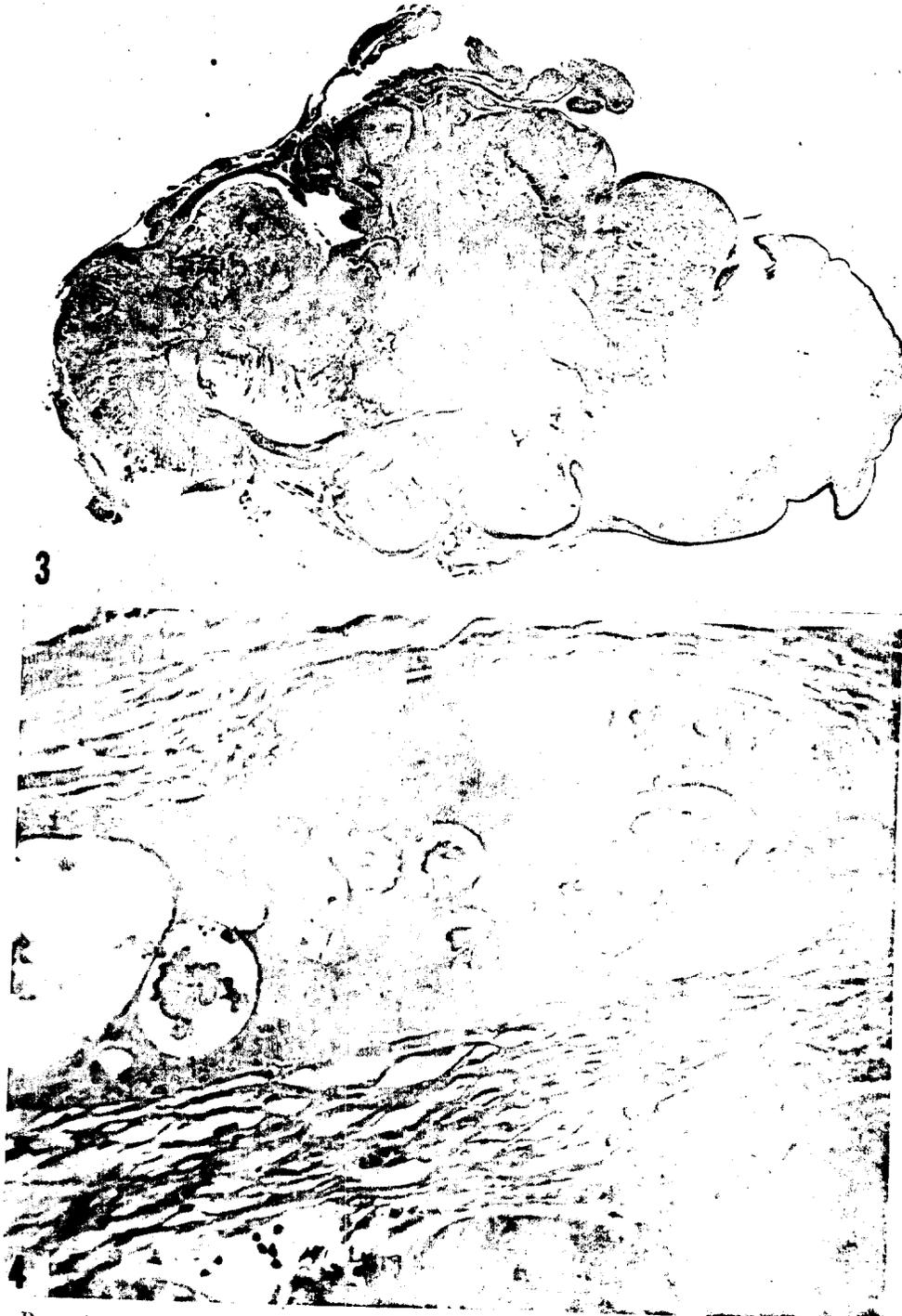


FIGURE 5.—Mouse (female) No. 495 51, age 596 days. Diet: basal plus cottonseed oil heated in nitrogen atmosphere at 320° C. for 2½ hours with iron filings. Extensive papilloma and squamous-cell carcinoma of forestomach spreading in submucous lymphatics to whole of lesser curvature of the stomach including the pars glandularis. Direct spread of carcinoma involved regional lymph nodes and small intestine. × 6

FIGURE 6.—Mouse (female) No. 495 51 (same as fig. 5), age 596 days. Tendency to central necrosis of tumour strands gives pseudo-acinar appearance. × 120

FIGURE 7.—Mouse (female) No. 495 51 (same as figs. 5 and 6), age 596 days. Well differentiated squamous and prickle cells can be seen in tumour invading muscularis propria of stomach. × 460

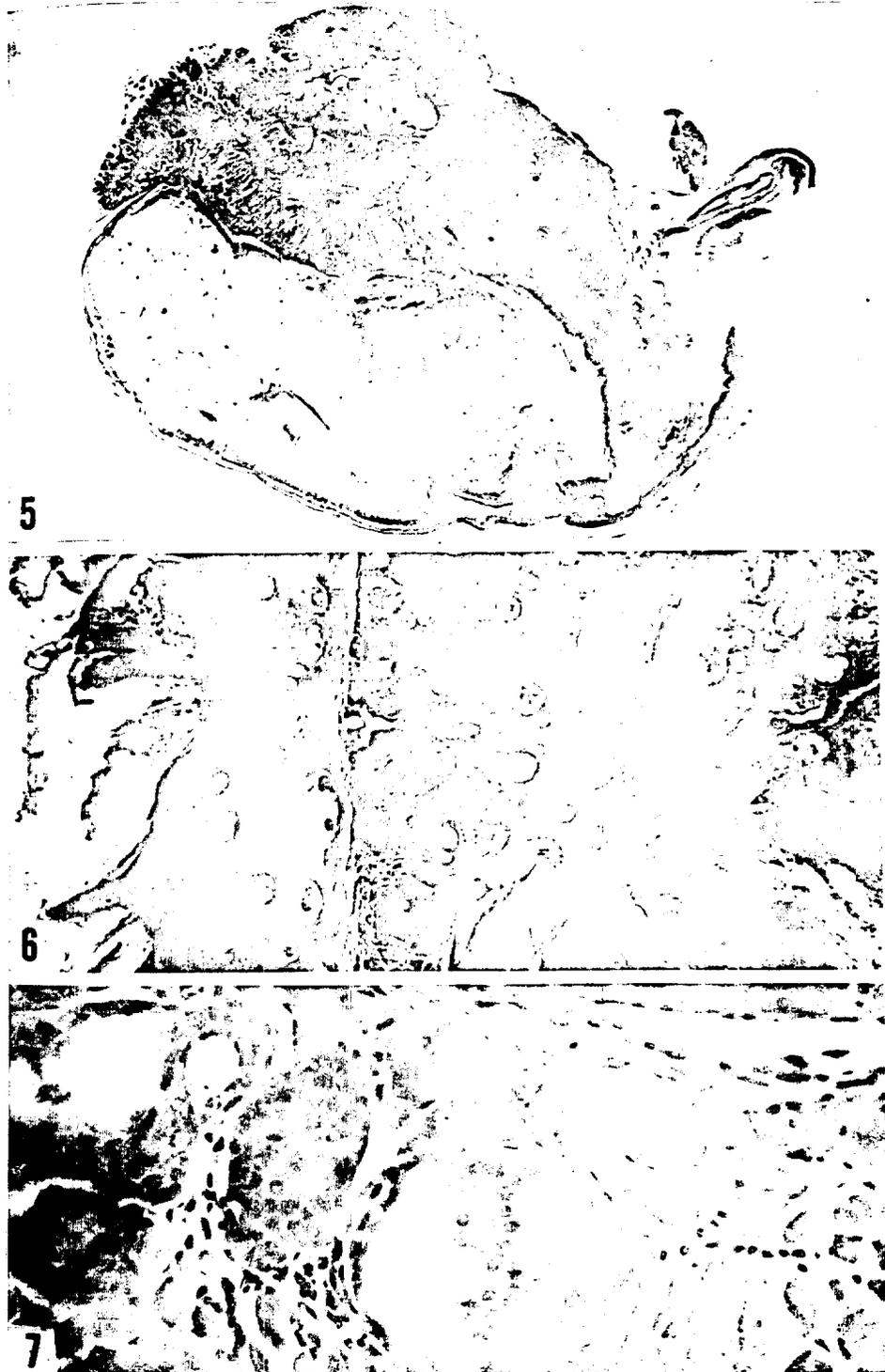
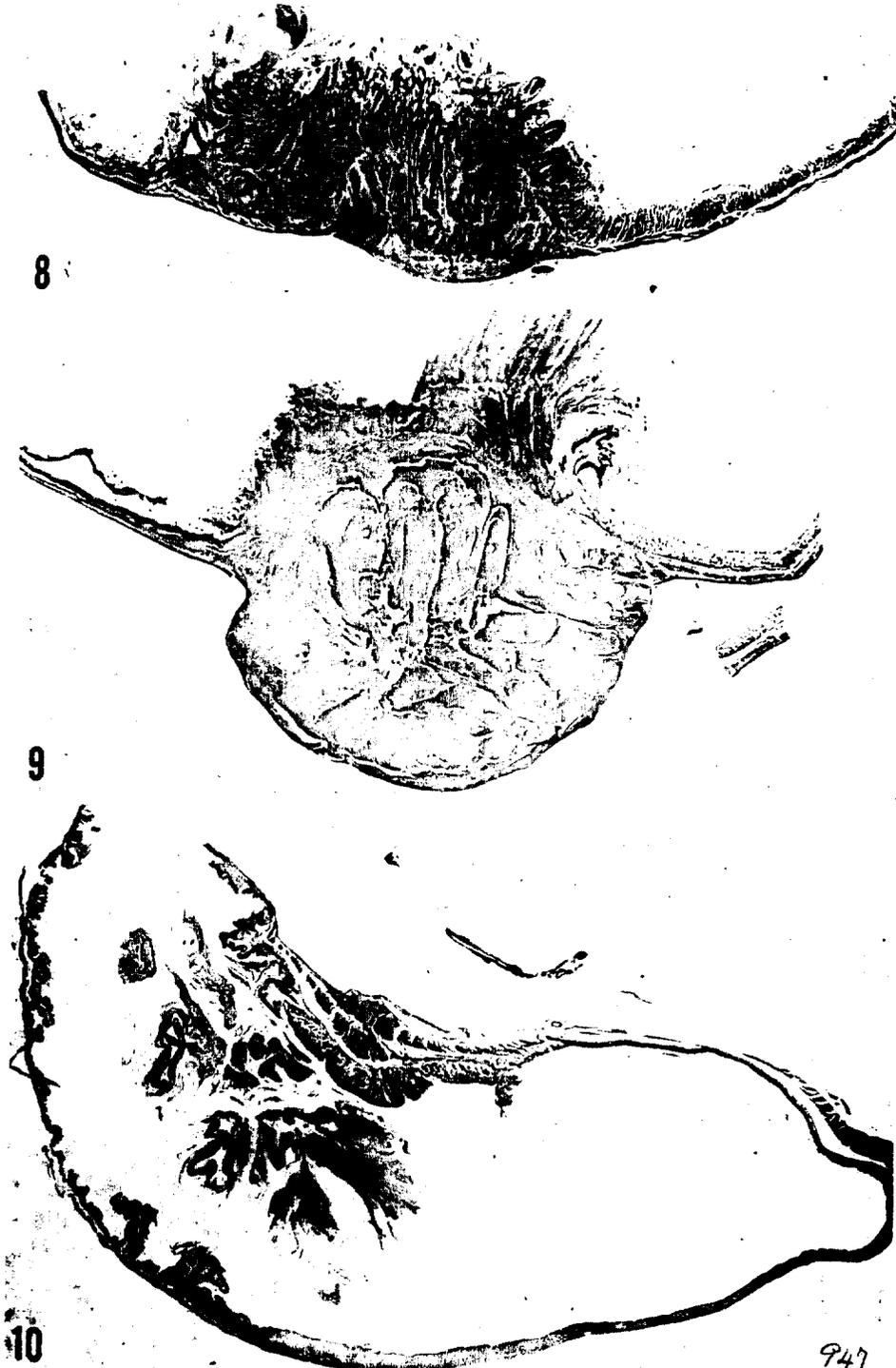


FIGURE 8.—Mouse (male) No. 75 52, age 754 days. Basal diet plus 5 percent cottonseed oil heated in nitrogen atmosphere at 320° C. for 2½ hours with iron filings. Squamous-cell carcinoma invading all coats of the stomach. X 10

FIGURE 9.—Mouse (female) No. 613 40, age 599 days. Injected 521 days previously in right flank with 0.2 ml. used domestic cooking fat. Nothing at site of injection; tumour on greater curvature of stomach projecting into peritoneal cavity and into lumen of stomach. Well differentiated keratinising papillary squamous-cell carcinoma penetrating all but the serous coat of the stomach. No metastases. X 12.

FIGURE 10.—Mouse (female) No. 61 52, age 764 days. Diet: basal plus 5 percent cottonseed oil heated in nitrogen atmosphere at 320° C. for 2½ hours with iron filings. Single large branching squamous papilloma and diffuse papilliferous hyperplasia of forestomach. Glandular stomach normal. X 10



EFFECT OF SURFACE ACTIVE AGENTS ON THE LATENT ATPase
OF MITOCHONDRIA*

by

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Previous studies^{1,2} have indicated that DNP^{***}, DCA, microsomes or an acetone-soluble, water-emulsified fraction derived from the latter greatly enhance the respira-

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*** The following abbreviations have been employed: DNP, 2,4-dinitrophenol; DCA, desoxycholic acid; Ap, acetone-soluble fraction of microsomes; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; LAS, latent ATPase stimulation; Mw₃, thrice washed mitochondria.

References p. 465-466.

tion of mitochondria in systems devoid of added phosphate acceptor. Preliminary reports have indicated that, in the absence of oxidizable substrate, comparable levels of these same agents bring about a marked stimulation of mitochondrial ATPase activity^{3,4}, a process which has previously been referred to as "latent ATPase" stimulation⁵. This latter property has proved particularly suitable for adoption into an LAS assay greatly facilitating further studies of the acetone-extractable fraction of microsomes. It has thus been possible to show that the properties of this agent can be accounted for on the basis of its content of several free fatty acids, one of which, stearic, has actually been isolated and identified.

The LAS assay system has also provided a means of studying the kinetics of the interaction between mitochondria and the LAS agents. On this basis it has been possible to distinguish the responses to a group of surface-active agents, including the Ap extract, from those elicited by DNP. In addition, several common fatty acids have been surveyed and correlations established between structure and LAS activity.

METHODS

Materials

Microsomal and thrice washed mitochondrial fractions were prepared in 0.25 *M* sucrose from rat liver according to the method of SCHNEIDER⁶. The Ap preparation was extracted from the microsomal fraction with acetone and, following the addition of water, the resulting emulsion was concentrated *in vacuo* in order to remove the acetone⁷.

ATP was obtained from the Pabst laboratories as the disodium salt. Oleic, linoleic and linolenic acids were obtained from the Hormel Foundation. Elaidic acid was prepared from oleic acid by elaidization with nitrous acid. We are indebted to Dr. F. M. STRONG of the Biochemistry Department for gifts of *cis*- and *trans*-vaccinic acids and to Dr. ROBERT MEYER of the Chemistry Department for purified samples of palmitic, stearic, brassidic and hexacosanoic acids. All other reagents employed were of the highest grade commercially available.

Estimations

Inorganic phosphate was determined by the method of LOWRY AND LOPEZ⁸ and the colorimetric organic solids procedure of JOHNSON⁹ was employed to determine the concentration of solutions or suspensions of purified fatty acids. In the case of the crude biological extracts, total organic materials were expressed as their equivalent in stearic acid. Maintenance of fatty acid solutions or suspensions of known concentrations presents certain technical difficulties and the following procedure was employed. Stock solutions of fatty acids were prepared by dissolving in water with the aid of enough sodium hydroxide to bring the pH to 9.5. Before each experiment the stock solutions were clarified by heating if necessary, diluted with isotonic KCl to the strength desired, and used immediately.

For determination of LAS activities the test substance was placed in a chilled centrifuge tube and brought to a volume of 0.5 ml with isotonic KCl and sucrose in such a manner that the final assay system was 0.03 *M* with respect to salt and 0.15 *M* with respect to sucrose. A salt requirement for optimal ATPase activity in a similar system has been reported¹⁰ while the sucrose has been found necessary both to minimize ATPase activity in the controls, and to assure optimal ATPase development in response to at least one LAS agent, DNP⁵. The mitochondria prepared from 5 g rat liver were suspended in 20 ml cold 0.25 *M* sucrose and 5 ml 0.1 *M* sodium ATP, pH 7.4, was added immediately before use to serve as both buffer and substrate. With the aid of a hand-operated automatic pipette, 0.5 ml samples of this mixture were rapidly added to centrifuge tubes containing the substances to be tested for LAS activity. The tubes were then placed at ten second intervals into an oscillating rack in a water bath maintained at 30°. Following a routine incubation of 15 minutes, the tubes were withdrawn, placed in an ice bath and deproteinized immediately with 1.0 ml cold 10% perchloric acid and then centrifuged. Aliquots of 0.5 ml were taken for inorganic phosphate analysis.

RESULTS

Identification of the microsomal agent

It was first established that agents previously found to raise the respiratory rate of

References p. 465-466.

mitochondria in a system free of phosphate acceptors would exert parallel LAS effects. In Fig. 1 are shown the influence on mitochondrial ATPase of varying levels of the Ap extract, DCA, DNP and octanol, the latter being included as a representative of another class of compounds exhibiting surface activity.

It is evident that graded amounts of the Ap extract do not elicit proportional ATPase responses from mitochondria. In order to adapt the ATPase responses evoked by different preparations to serve as measures of their respective LAS activities it was necessary to run each test sample at several levels and then arbitrarily assign unit activity to that quantity which stimulated mitochondria to release 1.5 μ moles of inorganic phosphate from ATP under the assay conditions. Such values, coupled with figures obtained from the organic solids determination served as a basis for following specific LAS activities during purification. The initial acetone-extracted material of the microsomal fraction had a specific LAS activity of from eight to sixteen units/mg.

When the crude material was extracted from petroleum ether with 50% ethanol containing dilute ammonia and KCl, re-extracted into benzene following saturation with CO_2 , and finally chromatographed on a silicic acid column, specific activities as high as 64 could be obtained. On removal of solvents a low-melting waxy material resulted, which, by repeated recrystallization from 90% methanol yielded a crystalline solid melting at 64–66.5°. Infrared spectral analysis* identified this substance as stearic acid which was consistent with carbon-hydrogen analysis. The specific LAS activity of this sample, about 40, was somewhat higher than that of an authentic sample of pure stearic acid, 30 (see Table I). This implies some contamination by acids of higher activity. The specific LAS activity of the crude waxy solid, 64, similarly indicates that it must have contained large amounts of fatty acids with higher LAS activity than stearic.

Analysis of the crude extract by the reversed phase, partition chromatographic column of HOWARD AND MARTIN¹¹ indicated that only about 10% could be stearic acid, the bulk of it consisting of palmitic, oleic and linoleic acids¹². That fatty acids are responsible for the effects of the Ap extract on mitochondria was further confirmed by finding that oleic or stearic acid are capable of affecting mitochondrial respiration and phosphorylation in much the same manner as previously reported for the Ap extract.

* We are indebted to Dr. DONALD JOHNSON of the Chemistry Department for running the infrared spectrogram. His extensive experience with fatty acids allowed Dr. JOHNSON to distinguish between stearic acid and closely related structures.

References p. 465, 466.

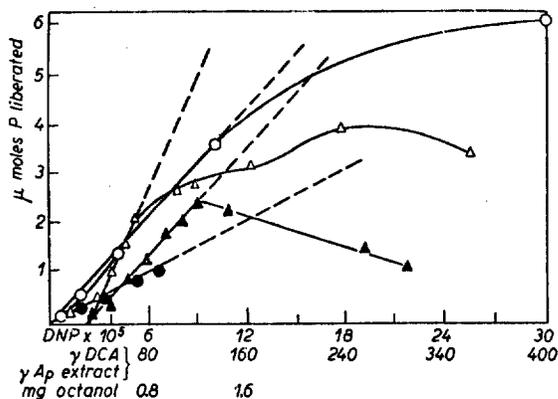


Fig. 1. Latent ATPase stimulation as function of agent concentration. For conditions see LAS assay under *Estimations*. \circ = Dinitrophenol; \triangle = Desoxycholate; \blacktriangle = Ap extract; \bullet = Octanol.

Correlation of the structure and LAS activities of fatty acids

When the LAS activities of the saturated fatty acids on either a weight or molar basis are plotted as a function of the chain length, Fig. 2, a definite optimum is exhibited centered around myristic acid. Comparison of fatty acids on the basis of type and degree of unsaturation (Table I) reveals that introduction of *cis*-unsaturation into the C₁₈ chain greatly enhances LAS activity. No further enhancement of activity results from additional unsaturation. Two singly *cis*-unsaturated positional isomers, Δ^9 -oleic and Δ^{11} -*cis*-vaccinic acids are of equal activity while their geometric isomers, elaidic and *trans*-vaccinic acids respectively, show no more activity than their saturated analogue, stearic acid. For purposes of comparison, the LAS activity of DCA and saponin are also included in the table.

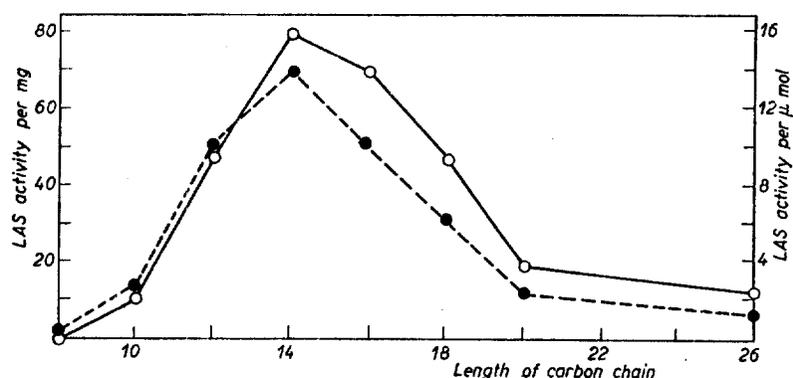


Fig. 2. Latent ATPase-stimulating activities of saturated fatty acids as function of carbon chain length. ○ = Activity on mg basis (scale on left); ● = Activity on μmole basis (scale on right).

TABLE I
EFFECT OF UNSATURATION ON LATENT ATPASE-STIMULATING ACTIVITY OF C₁₈ FATTY ACIDS

Acid	Unsaturation	LAS units/mg	LAS units/μmole
Stearic	None	30	8.5
Oleic	9, <i>cis</i>	76	21.4
Linoleic	9, 13, <i>cis</i>	79	22.2
Linolenic	9, 13, 17, <i>cis</i>	80	22.2
Vaccinic	11, <i>cis</i>	72	20.4
Elaidic	9, <i>trans</i>	32	9.0
Vaccinic	11, <i>trans</i>	32	9.0
DCA		60	24
Saponin		0.4	

Kinetic aspects of mitochondrial ATPase stimulation

The ease with which large numbers of determinations could be simultaneously conducted made the ATPase assay system suitable for studying the kinetics of the mitochondria-LAS agent interactions. In Fig. 1 the liberation of inorganic phosphate from ATP by a fixed quantity of mitochondria has been expressed as a function of the concentration of the added LAS agent. In Fig. 3 the LAS agent has been held constant at a level of approximately 1 unit and the quantity of mitochondria was varied. In

References p. 465; 466.

order to find out whether or not the phosphate liberated under the assay conditions represented true rate values, other experiments were conducted in which the levels of both mitochondria and LAS agents were fixed and the time of incubation was varied. These are summarized in Fig. 4.

The situation existing for DNP is the simplest for analysis and may be summarized as follows: (a) Added increments of DNP produce linear responses until the mitochondria approach full activation (Fig. 1). No lag phase occurs. (b) At $3 \cdot 10^{-5} M$ DNP (a concentration which activates only 15% of the potential ATPase activity, Fig. 1), the liberation of inorganic phosphorus is a linear function of the amount of mitochondria added (Fig. 3). In other words, the proportion of the potential activity liberated by a given concentration of DNP remains fixed over a wide range of mitochondrial concentrations. (c) Except for a brief lag accountable for by the time required for the chilled reaction mixture to reach bath temperature, the rate of liberation of inorganic phosphate is linear with respect to time (Fig. 4). These facts lead to the conclusion that mitochondria can bind only a very small fraction of the total DNP present at concentrations producing LAS stimulation. Furthermore, the effects of DNP are both instantaneous and reversible. This conclusion is consistent with the findings of LOOMIS AND LIPMANN¹³ that the effects of DNP on mitochondria may be reversed by a simple washing procedure.

More complex phenomena are apparently involved in the action of the other agents tested. Octanol evokes a linear response up to the highest concentration permitted by its limited solubility in water (Fig. 1). In Fig. 3, however, the octanol curve displays an unexpectedly high stimulation of ATPase at the lower levels of mitochondria. A dilution effect has also been reported for the low ATPase activity exhibited by mitochondria unstimulated by LAS agents⁵. In the latter case, however, the effect produced was in the opposite direction in that ATPase was disproportionately diminished when the mitochondria were more dilute⁵.

Extensions of the linear portions of both the DCA and the Ap extract curves in Fig. 1 fail to intersect the origin, indicating that mitochondria can bind a limited quantity of these agents without exhibiting any LAS response (difference from DNP). This lag phase also obtains for DCA in a respiratory system as shown in Fig. 5. Once the critical limit is exceeded the mitochondria enter a phase in which additional LAS agent increments produce linear LAS responses. In Fig. 3, the leveling and eventual falling off of the curves at the higher enzyme concentrations (not apparent for DCA within the limited areas graphed) are likewise consistent with the interpretation advanced for the lag phase, *i.e.*, inert binding of LAS agents.

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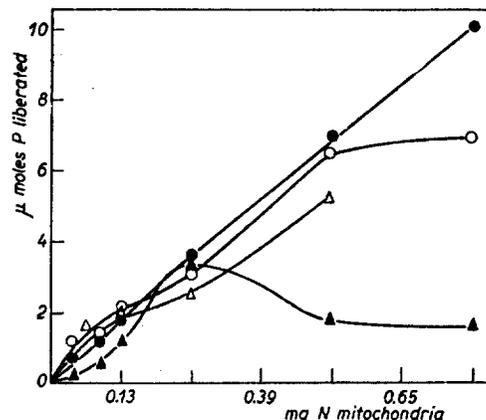


Fig. 3. Latent ATPase stimulation as function of quantity of added mitochondria. Conditions as in Fig. 1 except the amount of agent added was fixed at approximately the equivalent of 1 LAS unit and the quantity of mitochondria added varied. ● = Dinitrophenol; ○ = Desoxycholate; ▲ = Ap extract; △ = Octanol.

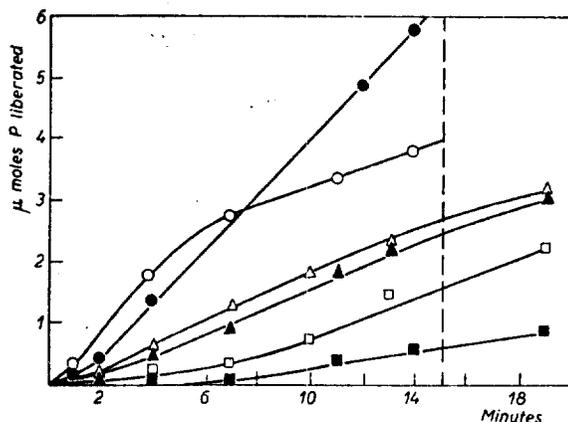


Fig. 4. Rate of ATP hydrolysis by mitochondria activated by various agents as function of time. Conditions as in Figs. 1 and 3 except that levels of mitochondria and added agents were both fixed and samples withdrawn periodically for inorganic phosphate analysis. The vertical line at 15 minutes indicates the amount of liberated phosphate corresponding to the standard assay conditions. ● = $10^{-5} M$ Dinitrophenol; ○ = 100γ Desoxycholate; ■ = 40γ Desoxycholate; Δ = 70γ Oleate; ▲ = 2.8 mg Octanoate; □ = 1.4 mg Octanoate.

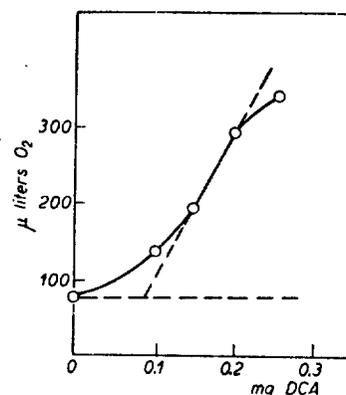


Fig. 5. Influence of desoxycholate on mitochondrial respiration. Each Warburg vessel contained in addition to the indicated amounts of DCA: 0.005 M α -ketoglutarate; 0.0013 M phosphate; 0.005 M $MgCl_2$; 0.025 M KCl; $10^{-5} M$ cytochrome c ; 0.0017 M ATP; 0.14 M sucrose; and 1.0 mg mitochondrial nitrogen. The final volume was 3.0 ml, pH 7.4, and temperature 30° . The gas phase was air and 0.2 ml 2 N NaOH was present in the center well. The figures on the ordinate give the oxygen consumed during a one-hour interval.

It can also be seen from Fig. 1 that the maximal ATPase activity which DCA or Ap can induce is never as great as that produced by DNP. Once the maximal level is reached, further additions of DCA or Ap depress ATPase activity. In this inhibitory phase, mitochondria do not respond to DNP.

Despite the aforementioned similarities in properties between DCA and fatty acids, significant differences do exist. Under the conditions described for Fig. 1, DCA induced higher maximal ATPase than did fatty acids. The response pattern of DCA at the lower mitochondrial levels of Fig. 3 resembles that of octanol rather than that of the Ap agent.

Influence of Mg^{++} on fatty acid stimulated ATPase

Since Mg^{++} has been shown to influence ATPase activity under a variety of conditions¹⁴, the effect of this ion on the LAS activity of representative fatty acids was investigated. According to Table II, the addition of 0.005 M Mg^{++} increases the ATPase activity induced by octanoic acid but fails to stimulate systems containing the higher fatty acids. The activity of stearic acid is actually lowered by Mg^{++} but this could result from the complexing of higher fatty acids by this cation. A clear difference is thus established between the properties of mitochondria under the influence of higher fatty acids and aged mitochondria which are markedly stimulated by added Mg^{++} ¹⁴. Both treatments result in development of latent ATPase activity and decreased phosphorylation efficiency² but while mitochondria treated with long chain fatty acids appear inert toward Mg^{++} , the ATPase activity of the aged mitochondria is greatly increased by the addition of Mg^{++} . These data, coupled with the differences

References p. 455, 466.

established in earlier kinetic studies suggest that the higher and lower members of the homologous fatty acid series affect mitochondria in qualitatively different manners.

TABLE II
EFFECT OF MAGNESIUM ON LATENT ATPASE STIMULATION BY FATTY ACIDS

Fatty acids added	μg	$\mu\text{moles P liberated}$		
		No Mg^{++}	0.0015M Mg^{++}	0.005M Mg^{++}
Octanoate	1200	2.2		3.8
	800	1.7		3.1
	600	1.5		2.1
	400	1.2	1.6	1.9
	200	0.9		1.0
Myristate	24	3.4		3.1
	16	0.9	1.5	1.2
	11	0.3	0.7	0.4
	8	0.1		0.4
Stearate	40	2.2	2.2	1.4
	30	1.5	1.5	0.9
	20	1.0	1.0	0.5
	10	0.3	0.6	0.3
Oleate	18	1.5		1.5
	13	1.0		0.9
	9	0.3		0.6
None		0.4	0.5	0.6

Conditions for hydrolysis of ATP by mitochondria as described under METHODS.

DISCUSSION

It has been suggested that lowering of the efficiency of mitochondrial oxidative phosphorylation could influence the regulation of cellular processes¹⁵. Among the classes of substances known to uncouple phosphorylation, *e.g.*, dyes¹⁶, thyroxine^{17, 18} and its analogs¹⁹ and other substituted phenols²⁰, fatty acids alone constitute a major component of all protoplasm. This suggests that specific cellular mechanisms may function to limit the level of fatty acids permitted to exist unesterified. Extraction of quick-frozen whole liver has yielded only small amounts of free fatty acids and thus the higher concentrations which were obtained from the microsomal fraction probably arose during the separation procedure through the action of the esterase which is localized in these particles²¹. Nevertheless, in view of the small amounts of fatty acids capable of altering the properties of mitochondria and indications that this effect is reversible*, the possibility that this interaction has physiological implications cannot be ruled out.

Inasmuch as the activation of latent ATPase represents an alteration of mitochondrial properties, the LAS activity of free fatty acids is an ever present complica-

* Mitochondria whose P/O ratio has been lowered by fatty acids will recover at least part of the lost phosphorylation efficiency if respiration is continued long enough to reduce the free fatty acid concentration.

tion in studies of the metabolism of these substances. This factor was recognized at an early date by LEHNINGER²². However, owing to practical considerations, much subsequent work on the metabolism of fatty acids has been done at substrate levels which the present quantitative study indicates capable of altering mitochondrial properties. Moreover, as indicated by the stimulation of mitochondrial respiration produced by the unmetabolizable DCA, increases in respiratory rate in mitochondria produced by addition of fatty acids are not necessarily related to their metabolic utilization when other oxidizable substrates are present.

The sigmoid mitochondrial ATPase response curves of Fig. 2 are reminiscent of similar patterns obtained in studies of the effects of hemolytic agents on erythrocytes. Suggested mechanisms for the latter phenomenon have resulted from kinetic studies²³ and a similar approach might yield useful information about the mitochondria-detergent interaction and the nature of the membrane or membrane-like structures of mitochondria.

DE DUVE *et al.*²⁴ have shown that besides erythrocytes and the ATPase-bearing granules, acid phosphatase-bearing granules termed lysosomes which are normally present in the classical mitochondrial fraction, constitute a third biological structure sharing the properties of extreme sensitivity to osmotic environment, mechanical disruption, aging and the action of detergents. The properties of these structures can be distinguished, however, on the basis of their sensitivity toward saponin. The low LAS activity, 0.4, found for this agent contrasts with its known hemolytic properties and its action on lysosomes²⁵. Saponin presumably exerts its effects by virtue of its specific chemical affinity for cholesterol, *cf.*²⁶, which suggests that although cholesterol constitutes a major constituent of mitochondria²⁷, it is not integrally associated with those structures involved in maintaining the latency of mitochondrial ATPase.

SUMMARY

In previous work a heat-stable, acetone-soluble fraction from rat liver microsomes was found to stimulate the respiration of mitochondria. By means of an assay system based on the activation of latent mitochondrial ATPase, the active component of the microsomal extract has been identified as free fatty acids.

Among the saturated fatty acids, myristic exhibited the greatest activity which diminished progressively as the chain was lengthened or shortened. Introduction of a *cis* unsaturated bond, but not of a *trans* unsaturation, enhanced activity.

During the interaction, the higher fatty acids or DCA are tightly bound by the mitochondria in contrast to DNP which activates latent ATPase without becoming appreciably bound.

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scientific data concerning the nutritive value of heated oils and fat.

Heated oils have been shown to be poorly absorbed¹, to produce cancerous tumours² and to cause paralysis resembling that due to vitamin E deficiency³. Thermal polymerization was found to develop toxic products responsible for growth depression⁴. Kaunitz⁵ stated that oxidized fats destroyed vitamins in the diets and thus caused retardation of growth. Chalmers⁶ found that injections of heat-polymerized cotton-seed oil did not lead to the growth of tumours.

In most of the above investigations, the oils were heated in a current of carbon dioxide or nitrogen. In India, oils are heated in open pans for long periods and, further, the residual oil from a day's operations is supplemented with fresh oil and re-heated again. This may accelerate the oxidative and other changes. The work reported in this communication was undertaken to elucidate the nutritive value of some of the commonly used oils when they were fed after heating.

Groundnut, sesame and coconut oils were heated in an open iron pan at 270° C. for 8 hr. They were then incorporated into synthetic diets to give 15 per cent fat, and fed to albino rats. There were six groups of six rats each, five weeks old and weighing 40-50 gm. The two groups of rats allotted to any particular oil (unheated and heated) were litter mates and were distributed with due consideration to weight and sex. The diet employed had the following composition: fat, 15 per cent; casein, 12 per cent; sugar, 10 per cent; salt mixture, 4 per cent; and starch, 59 per cent; and the vitamin supplements were: thiamine, 15; riboflavin, 60; pyridoxine, 10; niacin, 10; calcium pantothenate, 50 (all in mgm. per kgm. of the diet); and choline, 1 gm. Vitamins A and D were given in the form of two drops of 'Adexoline' twice a week per rat. The rats were housed in independent cages. The vitamin supplements were added to the diet every day, so as to prevent their destruction by the heated oils. Weekly growth records were maintained. At the end of the sixth week, four rats from each group were opened under chloroform, and liver, spleen, stomach and kidney were removed and weighed. The liver fat was also estimated. The observations are presented in Table 1.

Table 1. INFLUENCE OF HEATING THE OIL ON ITS NUTRITIVE VALUE AND FAT DEPOSITION IN THE LIVER

	Average gain per rat per week (gm.)	Feed efficiency	Liver weight as percentage of body weight	Percentage fat in liver
Groundnut oil:				
Unheated	13.0 ± 0.5	1.1	3.8 ± 0.3	3.9 ± 0.3
Heated	5.0 ± 0.9	0.67	5.7 ± 0.6	7.1 ± 0.4
Sesame oil:				
Unheated	10.0 ± 0.8	1.0	4.2 ± 0.7	4.8 ± 0.3
Heated	4.3 ± 0.7	0.7	6.0 ± 0.5	7.8 ± 0.3
Coconut oil:				
Unheated	11.0 ± 1.1	1.1	4.3 ± 0.3	4.5 ± 0.3
Heated	4.5 ± 0.7	0.7	5.8 ± 0.4	7.0 ± 0.3

Nutritive Value of Heated Vegetable Oils

Much of the fat consumed by human beings, particularly in India, has been heated, and the conditions of heating vary widely and also with the nature of the foodstuff into which the oil or fat is incorporated. It is therefore necessary to have

The results show clearly that in all three cases the heated oil has adversely affected the gain in weight. The feed efficiency, that is, the increase in weight per gm. of fat consumed, of the heated-oil group of rats is considerably reduced. There were no significant changes in the weights of stomach, kidney and spleen of the two groups of animals. These results are not, therefore, presented here.

It may be seen that the liver weights (fresh) of the heated-oil groups are significantly higher than the controls. The fat content of the livers of the heated-oil group is nearly twice that of the control groups. Heated oil has produced fatty infiltration of the liver. Further, the livers were badly damaged and congested, and were not of the normal colour.

When the heated oil was fed at 30 per cent level, all the rats died within a week, whereas the control rats were quite healthy. In another set of trials, when 15 per cent heated oil (heated at 230° C.) was used in the diet, severe jaundice occurred and four of the six rats died in the sixth week of the experiment. The exact changes brought about by heating the oil and also the other aspects of the problem are being studied.

Our thanks are due to Prof. K. V. Giri for his interest in the investigation.

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Cessation of Tillering in Young Grass Plants

DURING the early seedling growth of *Lolium perenne* plants in pots, Cooper¹ noted that the number of tillers per plant increased exponentially with time until some date 60–100 days after sowing, when the initial exponential growth was sharply curtailed and tiller production ceased. This phenomenon has been observed in experiments in which the chief concern

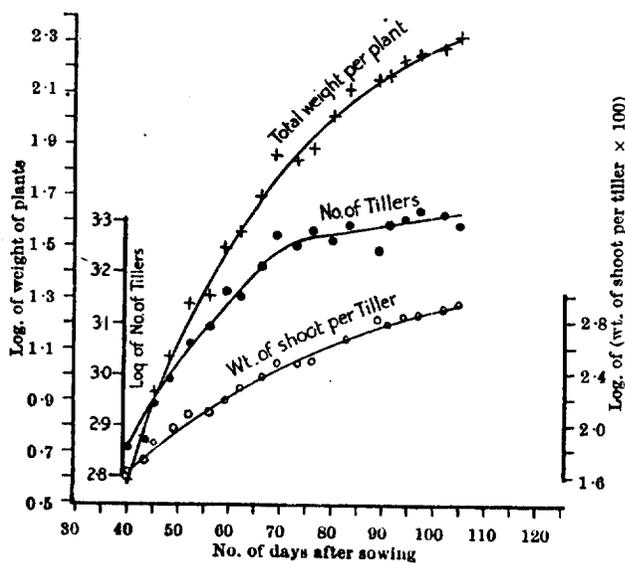


Fig. 1

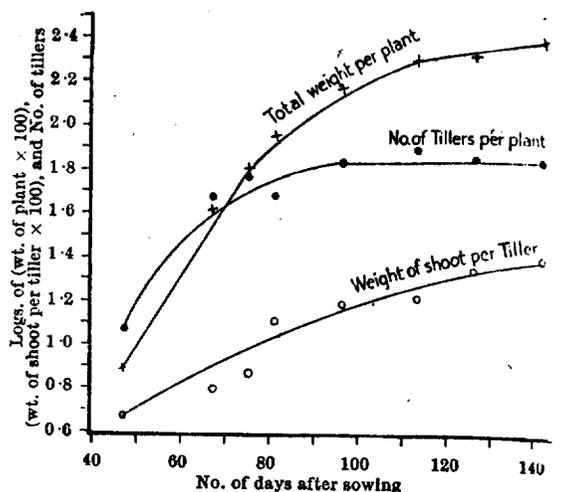


Fig. 2

was with growth as measured by increase in weight. The results obtained in a field experiment where the plants were growing in a sward are summarized in Fig. 1. In this experiment thirty plants of each of twenty-two strains representing seventeen grass species were dug up twice a week, washed free of soil, the tillers counted and then dried and weighed. Each point in the figure is derived from the measurement of 660 plants. In the majority of strains tiller production ceased about the seventieth day after sowing. No sudden change occurred in the rate of increase of the plants (roots plus shoots) or in the rate of increase of the weight of shoot per tiller, that is, the weight of a tiller. Other details of the experiment have been published elsewhere².

In another experiment, plants of *Lolium perenne* S.24 were grown in whalehide pots of 6,700 ml capacity. An equal number of pots containing one, two or three plants were taken at intervals, the plants washed free from soil and then treated as in the other experiment. Usually thirty pots were dealt with on each date. The results are presented in Fig. 2 as the mean of the plants measured on a particular date. Tiller production ceased about the hundredth day after sowing. As in the field experiment, there was no cessation of dry-weight increase comparable to the cessation of tiller production, although a gradual reduction in this rate did occur. The weight of shoot per tiller increased at the same rate throughout the period of the experiment.

This state of inaction of tillering would appear to affect chiefly the meristematic tissues concerned with the production of new tillers; the more mature tissues are unaffected and continued to grow, that is, to gain in weight without any sudden change in the rate of increase for some considerable time after the cessation of tiller production. This is in accordance with Cooper's³ findings that rate of leaf production on the main tiller was unaffected by cessation of tillering.

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TESTING CARCINOGENICITY OF EDIBLE OILS.

(Part) II.

MUSTARD OIL.

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THE mutagenic property of the mustard oil (Allyl isothiocyanate) was reported long back by Auerbach and Robson, 1944; Sikka and Swaminathan, 1956; Swaminathan and Natrajan, 1956, 1959. These workers have also described tumour production by mustard oil in allium root tips. This oil is widely used in Bengal and U.P. in the diet and is also commonly used for massage. To investigate the possible carcinogenic effect of mustard oil on animal tissues, a series of experiments were undertaken on laboratory mice.

MATERIAL AND METHOD.

Two samples of mustard oil were used for this study. One was obtained from the Indian Agricultural Research Institute, New Delhi, and the other was a locally available preparation - tinned mustard oil (Raj brand) from Raj Oil Mills, Bombay.

A total number of 86 hybrid mice (XVII × C57 black) was used as test animals. The treatment was started between the age of 10 and 12 weeks and the animals were observed till death or were sacrificed when they looked weak and emaciated.

Routes of administration. - The oil was administered by four different routes :

- | | |
|----------------------------------|---------------------------------|
| (i) Cutaneous application | (ii) Subcutaneous injection |
| (iii) Intraperitoneal injection. | (iv) Feeding by stomach tubing. |

Experimental groups were arranged as follows : -

To test mustard oil sample received from I.A.R.I., New Delhi :

Group I. - Fourteen mice were fed 0.05 ml. of oil daily (except Sundays) by stomach tubing for 3 months, and observed till death.

To test Raj brand mustard oil :

Group II. - Daily cutaneous applications of oil were given by No. 5 camel hair brush on the interscapular region of 12 mice.

Group III. - Croton oil was used as a co-carcinogen along with mustard oil in a group of 12 mice. Cutaneous application of mustard oil was given four days per week and 3 per cent croton oil in liquid paraffin was given once a week. Both the groups II and III received the treatment till death.

Group IV. - Four subcutaneous injections of 0.5 ml. of mustard oil were given to 12 mice at monthly intervals. Total dose was 2 ml. per animal.

Group V.—Five intraperitoneal injections of 0.2 ml. of oil were given to 13 mice at monthly intervals. Total dose was 1 ml. per animal. The animals from groups IV and V were kept under observation till death.

Group VI.—Ten mice were fed daily 0.05 ml. oil throughout their life except on Sundays.

Group VII.—An untreated control group of 13 mice was observed till death.

These animals were sacrificed when they appeared weak and emaciated. The site of treatment and all visceral organs including gonads were observed for gross abnormalities. Suspected tissues and the treated site were fixed for histopathological studies.

EXPERIMENTAL FINDINGS.

In the cutaneous application groups (groups II and III) no epidermoid tumour was developed. Skin was normal. Slight to moderate epidermal hyperplasia was seen in group III, where co-carcinogen was used.

In the subcutaneously injected group (group IV), deposition of oil or its metabolites was seen as observed before in the animals injected with pea-nut oil (Gothoskar and Ranadive, 1965). No tumour of subcutis was developed during 100 weeks of observation period.

In the intraperitoneally injected group (group V), no lesions were seen in the visceral organs.

In the mice with continuous feeding of oil (group VI) one of the ten mice developed papilloma of the stomach wall at the cardiac end. In the mice fed with oil, supplied by Indian Agricultural Research Institute, New Delhi (group I) for three months, three of the fourteen mice developed stomach papillomas (Plate LX). In untreated control mice, also, one of the thirteen had a papilloma of the stomach wall. Microscopic study of the liver lesions found in this series showed fatty degeneration, necrosis and patches of inflammatory cells. Such liver lesions were also present in the untreated control mice. Other abnormalities found in this series were atrophic spleen and enlarged lymph nodes (*vide* Table).

DISCUSSION.

Experimental testing of mustard oil administered by four different routes, namely cutaneous application, subcutaneous injection, intraperitoneal injection and oral feeding, was undertaken and the animals were observed for sufficiently long period of over 2 years. Few papillomas of the stomach wall, inflammatory lesions of the liver, atrophy of spleen and enlargement of lymph nodes observed in some experimental groups of mice were also found in the untreated mice. There was thus no evidence of any malignancy at the site of treatment or in the remote organs, that can be attributed to the administration of oil. These observations confirm the findings of Visser and Ten Seldam (1938) and of Larionow and Soboleva (1938) on albino mice (quoted by Hartwell, 1951).

TABLE.

Summary data of mustard oil testing for carcinogenicity

Sample of oil.	Route of administration.	Number of animals.	ABNORMALITIES :					Malignancy.
			Skin.	Liver (necrosis and fatty changes).	Spleen (atrophy).	Stomach (papilloma at the cardiac end).	Lymph node (enlarged).	
I.A.R.I. New Delhi sample.	Control	13	..	1	6	1	6	..
	Oral administration for 3 months	14	1 Papillomatous growth on eye lid	2	7	3	6	..
Raj Brand, Bombay.	Cutaneous application	12	..	1*	1	..
	Cutaneous application with co-carcinogen	12	6 Hyperplastic	..	1	..*
	Multiple subcutaneous injection	12	12 oil deposition	..	4	..*
	Multiple intraperitoneal injection	13	3	..*
	Continuous feeding	10	2	1	2	..

* Stomach wall not examined.

The mutagenic and tumorigenic activity of the mustard oil as reported by Swaminathan and Natrajan (*loc. cit.*) appears to be specific to plant tissues only. Testing oil for the mutagenic property direct on animal cells would perhaps throw more light on the subject.

SUMMARY.

Mustard oil has been tested for its carcinogenic activity on XVII × C57 (black) hybrid mice by four different routes : (i) Cutaneous application, (ii) subcutaneous injection, (iii) intraperitoneal injection and (iv) oral administration. There was no evidence of malignancy due to oil at the site of treatment or in the remote organs even though the animals were observed for their complete life span of over two years.

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**THE EFFECT OF VARIOUS FATTY ACID FRACTIONS OF
COCO-NUT OIL ON CALCIUM METABOLISM IN
NORMAL GROWING RATS.**

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THE fact that fat plays a fundamental rôle in influencing the mineral metabolism which is of considerable nutritional significance during the growth period has been sufficiently well established. A large volume of literature has accumulated on this important aspect of nutritional chemistry. Several recent publications indicate that the addition of fat to a fat-free diet has a favourable influence on calcium utilization. It has also been found that in some cases, due mostly to variation in the experimental conditions and diets, entirely different types of conclusions have been drawn. Among the first to investigate this relationship were Holt, Courtney and Fales (1920), who found that the utilization of calcium in infants was augmented by fat in the diet. Hickmans (1924) reported similar results. Knudson and Floody (1940) and Jones (1940) found that moderate amount of fat in the diet favoured utilization of calcium. An increased absorption of calcium and phosphorus from the intestine was observed by Boyd, Crum and Lyman (1932) when fat was added to the diet. Westerlund (1934, 1934a) investigating the influence of tripalmitin, triolein and tributyrin upon calcium metabolism in adult rats found that the feeding of tripalmitin caused negative calcium balances, while neither triolein nor tributyrin produced such an effect. It may be observed, however, that the level of calcium was kept extremely low so as to promote the highest degree of calcium utilization. French (1942) and French and Elliot (1943) fed adult rats with diets containing oleo oil at levels ranging from 0.28 per cent to 45.46 per cent. The diet contained sub-optimum level (0.3 per cent) of calcium with a calcium : phosphorus ratio of 1 : 1. They concluded from their results that oleo oil interfered to a slight extent with calcium retention, but their results were not very significant. Basu and Nath (1946) studied the effect of edible oils on

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the utilization of calcium in human beings and found that coco-nut oil caused negative calcium balance. The above conclusion of Basu and Nath was contradicted by Sadasivan (1950), who repeated their experiments but could not observe any negative calcium balance. Calverly and Kennedy (1949) studied the effect of pea-nut oil, coco-nut oil and hydrogenated cotton-seed oil on calcium metabolism and concluded that the inclusion of 5 per cent of each of the above fats in a normal diet increased the faecal excretion of calcium.

Several investigators have studied the relation of fat on the development of rickets in rats. Zucker and Barnett (1943) have reported that cotton-seed oil and its hydrogenated product showed anti-rachitic properties although it was not found possible to concentrate the anti-rachitic activity in the non-saponifiable fraction. From a series of investigations with butter-fat, lard, egg-oil, olive oil and the fatty acids obtained from butter and lard, Kon and Booth (1934) concluded that the anti-rachitic activity of the saponifiable fraction of fat was due to the fatty acids and not to any specific compound. They also found that on a high-calcium low-phosphorous diet calcification of bones was definitely aided by the presence of fat in the diet. This effect was not evident with a low-calcium high-phosphorous diet. Kandson and Floody (*loc. cit.*) fed rats a low-fat rachitogenic diet containing a known amount of vitamin D and 1.37 per cent of calcium with a calcium: phosphorus ratio of 5:11. They found that 5 per cent of hardened cotton-seed oil promoted better healing in rachitic rats. Larger amounts of fat were not so efficient. McDougall (1938) employing a diet deficient in calcium found that the rats were protected against the low calcium type rickets by including 4 per cent of fat in the diet.

That free fatty acids, such as palmitic and stearic acids, also affect the utilization of calcium to a considerable extent, was shown by Givens and Mendel (1917). They found that when such fatty acids as are utilized to a lesser extent were fed, the excretion of faecal calcium was increased. Palmitic acid and ethyl palmitate were found to produce negative calcium balance.

While fats most certainly seem to modify the utilization of calcium it would appear that factors such as the absolute level of calcium, the fat or fatty acid used and condition of the animal are important in determining the type of influence bestowed on the organism. In order to determine the effect of fat on calcium utilization by the different fatty acid fractions of fat, it seemed desirable to determine the effect of coco-nut oil and the various fatty acid fractions of coco-nut oil on calcium utilization in growing young rats.

EXPERIMENTAL RESULTS.

The coco-nut oil used in this experiment was quite fresh and pure. It was found to have the following physical constants: m.p. 22°C.; iodine value 11.8; acid value 1.8; and saponification value 258.2. The various fatty acid fractions of coco-nut oil were prepared as follows: The total fatty acids were liberated from coco-nut oil by saponification with alcoholic potash and acidifying with 10 percent sulphuric acid. The steam-volatile fatty acids were separated by steam distillation from the total fatty acids. The saturated and unsaturated fatty acids were separated from the remaining fatty acids by means of the lead salt separation method. The

melting point and the iodine value of the various fatty acid fractions were determined and the results are given in Table I:—

TABLE I.

Melting point and iodine value of fatty acid fractions used.

Fatty acid fraction.	Melting point.	Iodine value.
Steam-volatile fatty acid fraction ...	Liquid at room temperature	0
Unsaturated fatty acid fraction ...	" " "	74.8
Saturated fatty acid fraction ...	56°C.	3.4

The saturated fatty acid fraction must have an iodine value 'Zero'. Actually, however, it is found that all natural fats yield a solid acid fraction which has a small iodine value (usually about 3) due to contamination of the precipitated lead salts with small amounts of lead oleate which persists after crystallization.

Casein was freed from fat and calcium by treating crude casein with bile salts. The crude casein was suspended in water and a solution of sodium glycocholate in water was added. It was mixed thoroughly and kept in the refrigerator. Sodium glycocholate forms additional compounds with mineral salts and fat which are soluble in water. The casein was then filtered and dried.

The percentage composition of the diet used was as follows:—

	Per cent.
Casein (calcium and fat-free) ...	12
Corn starch ...	60
Sugar ...	10
Vitaminized starch ...	4
McCullum and Davis salt mixture ...	4

To this was added 10 per cent of each of the individual fat to be used: (i) coco-nut oil, (ii) steam-volatile fatty acid fraction, (iii) saturated fatty acid fraction, and (iv) unsaturated fatty acid fraction.

In the case of the diet with no fat, all the fat was replaced with corn starch. The vitaminized starch used in the experiment was so prepared that the addition of 4 per cent of vitaminized starch supplied the daily vitamin supplements of 40 γ of thiamine hydrochloride, 60 γ of riboflavin, 20 γ of pyridoxine, 20 γ of nicotinic acid and 40 γ of calcium pantothenate. Supplements of vitamins A and D were administered as 2 drops of Adexoline given by mouth to each rat every week.

Six rats weighing 80 g. each were placed on each diet. There were five groups of rats and each group was maintained on diets containing: (i) coco-nut oil, (ii) steam-volatile fatty acid fraction, (iii) saturated fatty acid fraction, (iv) unsaturated fatty acid fraction, and (v) no fat.

The faeces and urine were collected after a two-day preliminary period for 7 days. During the collection period the rats were kept in individual metabolism

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cages. The faeces were removed from the screen every day, stored in methanol and kept in the refrigerator. Bottles containing about 10 c.c. of the preservative containing equal amounts of toluene and concentrated hydrochloric acid were placed beneath each funnel for the collection of urine. Concentrated hydrochloric acid served to prevent the precipitation of calcium salts. After the collection period the funnels were washed with hot water acidified with hydrochloric acid and the washings were also included with the urine in the bottles. The unconsumed diet was collected every day in bottles and weighed after drying at 60°C. for 48 hours.

Calcium determinations were made on the diets and on samples of the faeces and urine using the methods of Morris, Nelson and Palmer (1931). The results of the effect of various fatty acid fractions of coco-nut oil on the utilization of calcium are given in Table II:—

TABLE II.

Summary of the effect of various fatty acid fractions of coco-nut oil on the utilization of calcium in young growing rats.

Data summarized.	No fat.	Saturated fraction.	Unsaturated fraction.	Steam-volatile fraction.	Coco-nut oil.
Number of rats ...	6	6	6	6	6
Average initial weight of rats, g.	87.3	85.1	83.5	87.3	86.0
Average final weight of rats, g.	106.1	103.0	96.6	100.3	99.0
Average gain, g. ...	18.8	17.9	13.1	13.0	13.0
Average amount of calcium present in the diet consumed, mg.	337.7	283.3	292.5	273.2	274.4
Average amount of calcium excreted in urine, mg.	3.33	4.0	11.4	9.4	8.36
Average amount of calcium excreted in faeces, mg.	178.4	140.4	83.6	169.3	121.4
Total amount of calcium excreted, mg.	181.7	144.4	105.0	178.7	129.7
Percentage of calcium utilization.	46.03 ±2.24	49.1 ±0.96	64.15 ±2.58	34.7 ±2.4	52.2 ±1.29

* Including the standard error of the mean calculated by the formula $\sqrt{\frac{\sum d^2/n-1}{n}}$ where 'd' is the deviation from the mean and 'n' is the number of observations.

INTESTINAL ACIDITY.

Boyd *et al.* (*loc. cit.*) presented evidence indicating that fats make the contents of the intestines more acid thereby increasing the absorption of calcium and phosphorus and facilitating bone formation. In order to determine the validity of this suggestion, the influence of different fatty acid fractions of coco-nut oil on the acidity of the intestinal contents was studied.

In order to determine the acidity of the intestinal contents that existed during the experimental period several precautions were taken. A time interval of five hours was allowed between feeding and killing of each rat. After a rat was killed by means of ether the entire gastro-intestinal tract was carefully removed and placed between two layers of wet gauze to keep it moist. The stomach along with the duodenum was then removed and its contents expelled into the small beaker of the Beckman pH meter. The pH of stomach and duodenum was determined together for the sake of experimental convenience. Four or five drops of distilled water were added and mixed thoroughly with the sample before the pH was determined. The jejunum was removed next and the contents treated in the same way. The third determination of pH was made on the contents of the ileum.

The slight dilution of each sample did not affect the hydrogen-ion concentration. The acidity of the contents of the stomach and duodenum, jejunum, ileum, in the rats fed various fatty acid fractions is given in Table III :—

TABLE III.
pH of intestinal contents.

Fat component.	Stomach and duodenum.	Jejunum.	Ileum.
No fat	5.0	6.46	6.73
Coco-nut oil	4.7	6.08	6.25
Saturated fatty acid fraction ...	4.4	6.04	6.64
Un-saturated fatty acid fraction ...	4.3	6.1	6.34
Steam-volatile fatty acid fraction ...	4.0	6.7	7.1

All the animals gained weight showing an increase of about 15 g. during the experimental period. The excretion of calcium in faeces would seem to depend on the melting point of the fat, coupled with the intestinal acidity produced by the fat or fatty acid used. Thus, of the three fractions of coco-nut oil calcium excretion was maximum with the saturated fraction and least with the unsaturated fraction. In general, urinary calcium varied inversely to faecal calcium though the variations were of smaller magnitude. The group of rats receiving fat-free diet consumed a larger amount of the diet than the others.

DISCUSSION.

Inclusion in the diet of 10 per cent fat or fatty acid fraction which has a definite nutritional function increased the utilization of calcium. Thus, the percentage of calcium utilized with a diet containing: (a) coco-nut oil was 52.2; (b) 10 per cent of unsaturated fraction was 64.15; (c) 10 per cent of saturated fraction was 49.1; and (d) no fat was 46.03. The results reported in this paper confirm the observations made by previous workers in that inclusion of fat in the diet increases calcium utilization. Calverly and Kennedy (*loc. cit.*), however, reported that addition of 5 per cent fat as coco-nut oil, pea-nut oil, or hydrogenated cotton-seed oil lowered calcium utilization. It may be mentioned that the results presented in this paper as also those of a number of others are quite in divergence with the observation of Calverly and Kennedy (*loc. cit.*).

The high utilization of calcium obtained with a diet containing no fat in his experiments cannot be explained.

Toller (1930) has outlined the following mechanism for the effect of fat on calcium utilization: Free fatty acids dissolve calcium phosphate with the formation of calcium soaps which are then absorbed. He also pointed out that if fats and phosphates were both low, calcium carbonate may be excreted in an alkaline stool. The form in which calcium was eliminated from the intestinal tract was, therefore, associated with the pH of the intestinal contents as a consequence of the effect of acidity upon solubility; the greater the acidity of the intestinal contents, the greater will be the utilization of calcium.

The decreased acidity of the intestinal contents, when rats were fed a diet free from fat, seems to be a factor in contributing directly to a lowered calcium utilization.

The work of Holt *et al.* (*loc. cit.*) may serve to indicate that the larger amounts of faecal calcium in rats fed the saturated fatty acid fraction of coco-nut oil is probably due to poor absorption of calcium stearate and calcium palmitate formed in the digestive tract. Hoagland and Snider (1943) have also reported on the poor absorption of stearic acid and palmitic acid. Boyd *et al.* (*loc. cit.*) reported 25 per cent and 33 per cent, respectively, for the utilization values of calcium stearate and calcium palmitate in young rats when the calcium intake was 37 mg. to 56 mg. The large amount of fatty acids present as soap in the faeces of these animals (Rao and De, 1951) would lend further support to substantiate this

percentage of calcium utilization in the case of rats fed coco-nut oil as the diet was 52.2 per cent. Calverly and Kennedy (*loc. cit.*) reported 49.1 per cent for calcium retention with a diet containing coco-nut oil but in their experiment the fat was given only at 5 per cent level. The excretion of free fatty acids in rats fed on the coco-nut oil diet was much smaller (Narayana Rao and De, 1951) so that it seemed improbable that such a reaction was a factor in the low percentage of faecal calcium noted in the group of animals fed on this diet. That calcium stearate and calcium palmitate were utilized by the rat at least to a limited extent was shown by Boyd *et al.* (*loc. cit.*). It would be reasonable, therefore, to assume that the amount of calcium stearate and calcium palmitate which would be absorbed on the coco-nut oil diet were absorbed to a considerable extent.

Bergeim (1926) demonstrated that calcium was excreted into the intestine. Hence, the presence of calcium in the faeces may be due to lack of absorption or increased excretion. The extent of excretion would depend upon the nature of the diet. Following a period of feeding calcium citrate, Steggarda and Mitchell (1946) found faecal calcium to be as much as twice the intake. From the data of the present studies it would be difficult to state whether the increase of calcium in the faeces of the rats given coco-nut oil was due to decreased absorption or increased excretion.

With the diet containing 10 per cent of unsaturated fatty acid fraction which contains about 83 per cent of oleic acid and the remaining linoleic acid, the percentage of calcium utilization obtained was 64.1. This high degree of calcium utilization may be explained by the fact that calcium oleate which is formed in the intestine is utilized to a large extent. Boyd *et al.* (*loc. cit.*) reported 90 per cent for the utilization of calcium oleate.

With the diet containing 10 per cent of steam-volatile fatty acid fraction the percentage of calcium utilization was 34.7. It is rather difficult to explain this observation. It may not, however, be due to a low utilization of the fatty acids as the lower fatty acids are generally utilized completely in the rat. The rats in this group developed diarrhoea. Although it is not possible to say at this stage whether the diarrhoea itself was brought about by the steam-volatile fraction, the low calcium utilization may be due to the poor absorption in the intestine under these conditions.

The utilization of calcium paralleled the acidity of the intestinal tract, the most efficient calcium utilization accompanying the most acid reaction. In the case of rats fed a diet containing the steam-volatile fraction, the pH of the intestinal contents tended to be on the alkaline side. This aspect requires further careful study which is in progress.

SUMMARY.

1. The inclusion of 10 per cent fat (coco-nut oil, saturated and unsaturated fatty acid fraction) in a normal diet increased the utilization of calcium in young growing rats.
2. The excretion of calcium in the urine was affected only indirectly by the presence of fat in the diet. When the faecal excretion of calcium was considerably increased there was a decrease in the urinary excretion of calcium.

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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 atherosclerotic, 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 methods

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, cellulose¹⁾ 12, cholesterol 5, salt mixture²⁾ 4, sodium cholate 2, sucrose 23, vitamin mixture³⁾ 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* (C901, Doivin type) lipopolysaccharide²⁾. 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 arbitrary 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.

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 (number of 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 %)	80 ± 19	40 ± 12	61 ± 16	355 ± 72	149 ± 30	493 ± 56	125 ± 18	111 ± 13
Lipoproteins (% α + α ₁)	31 ± 1.0	33 ± 1.5	30 ± 0.9	—	27 ± 1.5	20 ± 3.2	32 ± 2.0	37 ± 2.0
Lipoproteins (% α ₂ + β)	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	223 ± 7
Atherosclerosis, 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
Survival (%)	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 (number of 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* (% α + α ₁)	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

* 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 fibrous tissue. 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 $a + a_1$ 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 $a + a_1$ 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 erucic 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 clear relationship between the thrombogenicity and the atherogenicity of a fatty acid. Among the fatty acids examined, linoleic and linolenic acids were the least thrombogenic and also the least atherogenic, while palmitic and stearic acids were the most effective in these regards.

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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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 hyperlipemic 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. Tricaprylin 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 man¹. 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 fat². 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 lipemia⁷. 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 hypercholesterolemia⁸.

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The question now arises whether the well known effect of dietary fats on the production of thromboses and atherosclerosis⁹ 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 lipopolysaccharide¹³. 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 experiments¹¹, 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 clear-cut 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 conditions¹⁵, 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 animals 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 is 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 elsewhere¹³. 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 elsewhere¹³. In part 1, series 1 (Table 1) and 3 (Table 3), a *Salmonella typhosa* (0901, Bevin 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 previously¹³. 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 method¹⁸; the lipoproteins by paper electrophoresis post-stained with fat-red 7B according to our modification¹³ of the STRAUS AND WURM technique¹⁹. 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 veins²⁰. 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 given²¹, 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 reported²⁰. 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 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 a 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 I

INFLUENCE OF DIETARY FATTY ACIDS ON LEUKEMIA, CLOTTING TIME, THROMBOSIS, AND ATHEROSCLEROSIS, IN HOODED RAT

Results — mean \pm 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 \pm 12	169 \pm 7	135 \pm 5	149 \pm 5	173 \pm 5	168 \pm 10
	Serum cholesterol (mg/100 ml)	316 \pm 40	202 \pm 16	201 \pm 32	236 \pm 22	391 \pm 34	213 \pm 10
	Serum triglycerides (mg/100 ml)	69 \pm 12	33 \pm 6	24 \pm 6	43 \pm 10	96 \pm 11	35 \pm 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 \pm 22	178 \pm 12	143 \pm 8	137 \pm 11	128 \pm 9	164 \pm 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 \pm 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	Etched
Group		1	2	3	4	5	6	7
		<i>Weeks</i>						
Number of animals		11	13	9	11	6	8	12
Plasma clotting time (sec)	8	175 \pm 10	177 \pm 7	200 \pm 24	170 \pm 13	156 \pm 7	176 \pm 6	217 \pm 9
	12	200 \pm 30	186 \pm 9	151 \pm 11	162 \pm 10	148 \pm 9	162 \pm 8	164 \pm 7
	16	171 \pm 15	171 \pm 11	150 \pm 10	142 \pm 14	120 \pm 8	126 \pm 9	153 \pm 7
Serum cholesterol (mg/100 ml)	8	241	197	187	233	758	391	234
	12	410 \pm 43	296 \pm 21	538 \pm 73	370 \pm 67	1975 \pm 66	638 \pm 120	311 \pm 18
	16	655 \pm 92	493 \pm 47	1374 \pm 154	363 \pm 41	1530 \pm 256	648 \pm 54	290 \pm 8
Serum triglycerides (mg/100 ml)	8	59	42	28	76	139	96	43
	12	81 \pm 6	92 \pm 14	128 \pm 25	89 \pm 19	176 \pm 7	108 \pm 10	50 \pm 13
	16	91 \pm 5	79 \pm 6	257 \pm 8	107 \pm 9	270 \pm 26	163 \pm 25	71 \pm 3
Serum lipoproteins (percentage $\alpha_2 + \beta$)	8	54	53	53	54	59	62	54
	12	58 \pm 1.4	54 \pm 1.4	58 \pm 1.9	54 \pm 1.6	61 \pm 2.6	60 \pm 1.6	52 \pm 1.7
	16	58 \pm 2.3	57 \pm 1.6	67 \pm 3.2	56 \pm 1.5	64 \pm 2.8	59 \pm 1.7	51 \pm 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

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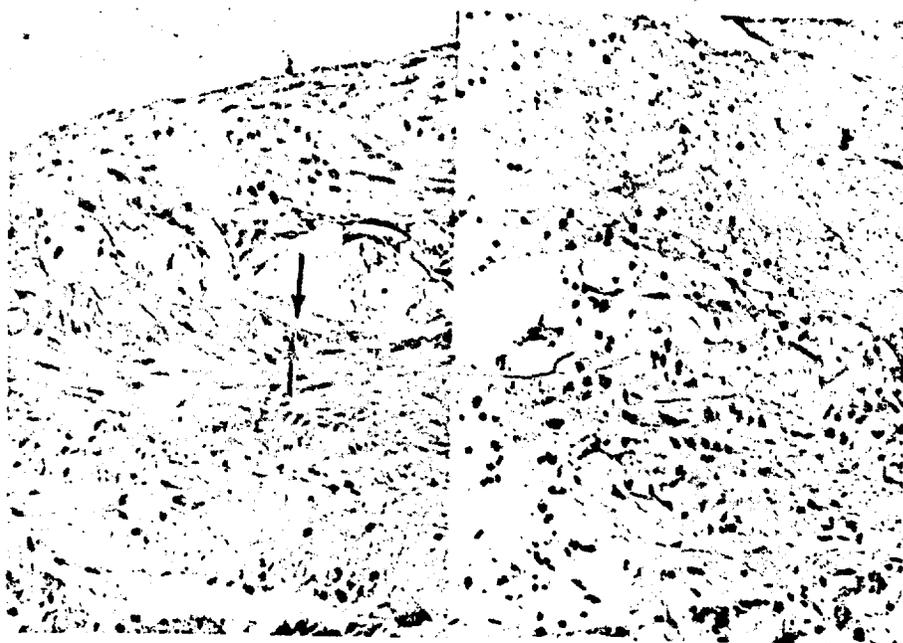


Fig. 1. On the left, a fibrous plaque with lipid material mostly located near the internal elastic membrane (arrows) on the wall of the valsalva sinus of a rat fed behenic acid. On the right, a fatty streak at the orifice of a coronary artery in a rat fed stearic acid. Here the lesion consists almost solely of foam cells with disruption of the elastic membranes. Hematoxylin-phloxine-saffron, $\times 220$.

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 $\alpha_2 + \beta$ 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 group 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, till 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 tricaprillin 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 rabbit²², dog²³ and rat²¹, 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^{20,24,25}. 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 \pm S.E. Diet composition in addition to the triglyceride: casein 11, cellulose 1, cholesterol 1, linoleic acid 1, salt mixture 4, sodium cholate 0.5, sucrose 33, vitamin mixture 1. Duration of the experiment: part 1, 11 weeks; part 2, 20 weeks. Group comparison: triglyceride 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, $P < 0.01$. Plasma clotting time, part 2, group 2 vs. 1, $P < 0.01$ and 3 $P < 0.001$.

		<i>Triolein 31</i>		<i>Tricaprylin 25</i> <i>+ triolein 6</i>		<i>Tristearin 25</i> <i>+ triolein 6</i>	
<i>Group</i>		<i>1</i>		<i>2</i>		<i>3</i>	
<i>Part 1</i>	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		
<i>Part 2</i>	Number of animals	14		17		14	
	Plasma clotting time (sec)	131	5	188	12	133	6
	Cholesterol (mg/100 ml)	1433	120	639	102	960	109
	Triglycerides (mg/100 ml)	214	21	106	6	233	15
	Atherosclerosis severity (0-3)	0.6	0.4	0.1	0.06	1.9	0.7

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 this 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 level²⁵. 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 3); 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 atherogenic¹¹. 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 atherogenic¹⁴. 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 reported¹⁴ 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 time²⁵.

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 cholesterol⁸. Finally, it is also known that, in man, oleic acid has no lowering effect on the serum cholesterol²⁷, 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 plaques²⁸. Nevertheless, certain epidemiologic studies have shown^{9,29,30} 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 superior¹⁷. 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 recently³¹ 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*^{10,11,22}, 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 absorbed¹⁶. 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 other

investigator¹² 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, Pickering¹ 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 clotting⁵; in addition, there is a strong possibility that the long-chain saturated fatty acids of the dietary fat are responsible for thrombosis⁶ 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 model⁹ 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 bacterial 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 show¹¹⁻¹³ that, under these conditions, as *in vitro*, the long-chain fatty acids were once again the most thrombogenic and that stearic acid appeared to be the most active among them. However, the very-long-chain fatty acids, such as behenic acids, were not thrombogenic, probably because of very poor digestibility.^{12, 13}

In these studies in rats, as in man,^{14, 15} palmitic acid was the most hypercholesterolemic of the fatty acids used, whereas stearic acid or short-chain fatty acids such as caprylic acid were much less hyperlipemic. As was also shown in man,¹⁶ only linoleic and linolenic acids, but not oleic acid, were hypolipemic.

Other investigators working on the relationship between the human diet and its lipemic effects¹⁵ recently suggested that people wishing to avoid coronary disease should substitute cacao butter for butter in the diet, because the former is richer in stearic acid, a long-chain saturated fatty acid that is much less hypercholesterolemic than palmitic, myristic and lauric acids. Judging from the results of work with blood platelets *in vitro*, such recommendations could be disastrous for coronary patients.

Inasmuch as our previous findings with dietary fatty acids seemed to have established the essential link between lipemia in man and the work with

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blood platelets and coagulation *in vitro*,^{12, 13} it was deemed necessary, because of the obvious implications, to make a detailed study under our experimental conditions of most of the common fats utilized in human nutrition. Our aims in these experiments were to verify: (1) whether the thrombogenic, atherogenic and lipemic effects of a dietary fat could be predicted from its composition in fatty acids; (2) to what extent the degree of lipemia as expressed by the serum cholesterol or triglyceride level could be a reliable indication of the thrombogenic or atherogenic capacity of a fat.

In addition, since we had not previously studied myristic and lauric acids, and a knowledge of their effect under our experimental conditions was essential for the present work on dietary fats, these fatty acids were studied here in a small experiment in comparison with caprylic, palmitic and stearic acids.

MATERIAL AND METHODS

Male Holtzman rats with an initial body weight of 150 to 170 gm were utilized. For each experiment, the exact number of animals actually utilized is shown in the corresponding table. The results of experiment 2 (table 2) and experiment 3 (table 4) represent the pooled data of several sets of experiments, with 6 animals per group in each set. These data were pooled because they were practically identical and resulted from experiments performed at approximately the same time and under comparable conditions. The rats were housed in a constant temperature environment and given, *ad libitum*, tap water and the hyperlipemic diet described in detail elsewhere.^{10, 11} In the tables are listed the ingredients composing the diets utilized in the corresponding experiment. The casein, cellulose (Alphacel), cholesterol, salt mixture (Wesson), sodium cholate, sucrose and vitamin mixture (vitamin diet fortification mixture) were obtained mixed in the right proportion by the supplier. The fatty acids utilized in experiment 1 (table 1) were also purchased from the same source (Nutritional Biochemicals Corporation). The animal fats utilized were ground and then melted in a steam bath; a sample of each of these tallows was analyzed by gas-liquid chromatography and the results are reported in table 3. Except for the coconut oil (Matheson, Coleman and Bell) and cacao butter, kindly supplied by Fry-Cadbury Ltd., all the fats and oils were commercially available and were therefore purchased from local stores. When the diets were prepared, isocaloric amounts of the fat (melted when necessary) were carefully mixed with the basic ingredients and then kept under refrigeration for not more than 2 weeks.

At the end of the feeding periods and following 17 hr of fasting, approximately 3 ml of blood were removed in siliconized syringes, equipped with 20-gauge needles, from the jugular vein of the rats, under light ether anesthesia, as reported in detail elsewhere.¹¹ To initiate thrombosis in experiments 1, 2 and 3 (tables 1, 3 and 4), a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide (0.3 mg per kg) in saline was injected by the same needle, after the last withdrawal of blood.

In each rat of the first three experiments, the plasma clotting time after recalcification was performed in duplicate, in plastic tubes (polycarbonate No. 2804, International Equipment Company, Boston), according to the technique already reported.¹¹ Triglycerides and total cholesterol analysis were performed on individual sera. The triglycerides were determined by the Van Handel method.¹⁷ In experiment 3 (table 4), Florisil (60 to 100 mesh, Fisher Scientific Company) was used, instead of zeolite as suggested by other investigators¹⁸, 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 m μ 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 Goodwin²⁰ utilizing only 0.5 ml of plasma, and the unesterified fatty acids by the technique of Itaya.²¹

In the thrombosis experiments, the time of death was recorded and the animals were autopsied. Survivals were killed at 24 hr. with chloroform. Except in experiment 2 (table 2), in which the lesions were graded according to a scale of 0 to 3 as described elsewhere,¹³ a quantitative evaluation of the thrombotic phenomenon could be obtained by weighing the liver before and after removing the red infarcts, as shown in figure 1. The severity of the thrombotic phenomenon was then expressed as the percentage in weight of infarcted liver.

In the atherosclerosis experiment (table 5), the animals were killed with chloroform after the last blood removal and the heart and the aorta were dissected as far as the common iliac arteries. The severity of the aortic fatty streaks was then graded according to a scale of 0 to 3 as already described,¹³ under binocular examination of the fresh aorta, opened longitudinally. Macroscopic readings were verified by routine histologic examination of the heart and aorta.

RESULTS

Experiment 1 (table 1). Depending on the fatty acid added to the already high fat diet, the results obtained differed markedly. Palmitic acid gave rise to by far the highest values for cholesterol and triglycerides; the next most hyperlipemic fatty acids were caprylic and myristic acids, followed by stearic acid; lauric acid was the least hyperlipemic. In group 5 fed stearic acid, the plasma clotting time was the shortest and the severity of thrombosis the highest; this was followed by the results for group 4 in which the clotting time was significantly longer and the thrombotic lesions were less severe, although not

TABLE 1

Experiment 1: thrombogenicity of various fatty acids

Results = mean \pm 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 \pm 172	156 \pm 24	116 \pm 8	2.0 \pm 1.1	9.3 \pm 2.3		
2. Lauric	12	6	401 \pm 33	111 \pm 29	198 \pm 17	2.3 \pm 1.6	12.7 \pm 3.4		
3. Myristic . . .	14	7	738 \pm 37	154 \pm 16	184 \pm 9	0	7.4 \pm 2.8		
4. Palmitic . . .	16	7	1153 \pm 135	258 \pm 39	143 \pm 8	8.2 \pm 3.6	3.3 \pm 1.5		
5. Stearic	18	7	528 \pm 74	130 \pm 15	121 \pm 3	14.5 \pm 6.3	11.4 \pm 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 \pm 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 \pm 23	148 \pm 6	155 \pm 7	2.1	100	4.2 \pm 0.7
2. Butter 38.....	12	585 \pm 80	135 \pm 4	185 \pm 12	0.6	30	18.1 \pm 2.3
10 weeks							
3. Butter.....	33	487 \pm 45	134 \pm 12	139 \pm 4	1.9	59	13.9 \pm 1.6
4. Lard 32 + water 6.....	12	459 \pm 25	115 \pm 6	148 \pm 8	0.8	58	14.2 \pm 2.7
5. Butter 32 + corn oil 6.....	24	340 \pm 21	94 \pm 9	164 \pm 11	0.4	46	16.7 \pm 1.8
6. Corn oil 32 + water 6.....	12	566 \pm 1	75 \pm 4	185 \pm 7	0.1	16	23.0 \pm 0.7

TABLE 3

Composition of certain fats in the commonest fatty acids

Selected values adapted from, "Fatty acids in animal and plant products," by V. R. Goddard and L. Goodall, Human Nutrition Research Division, United States Department of Agriculture, 1959.

Fatty Acids (Carbon Atoms)	Coconut Oil	Butter*	Cacao Butter	Bovine Tallow*	Lard	Chicken Tallow*	Horse Tallow*	Olive Oil	Arachis Oil	Corn Oil
Lauric (12).....	45.4	1.1								
Myristic (14).....	18.0	9.1		3.3		1.0	5.6			0.1
Palmitic (16).....	10.5	28.6	24.4	24.9	32.2	24.6	29.6	9.2	8.2	8.3
Stearic (18).....	2.3	14.6	35.0	16.9	7.8	6.9	4.9	2.0	6.2	3.4
Arachidic (20).....	0.4							0.2	4.0	0.4
Lignoceric (24).....									3.9	0.2
Palmitoleic (16:2H)...	1.3	2.7		5.3		6.5	6.0			1.2
Oleic (18:2H).....	7.1	33.0	28.2	46.3	48.0	45.3	31.3	83.1	52.9	30.0
Linoleic (18:4H).....	0.4	2.4	2.4	3.3	11.0	15.7	8.2	3.9	24.7	54.1
Linolenic (18:6H).....		1.4			0.6		15.0			0.6
Iodine index.....	7.8	33.4	37.0	48.3	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 \pm 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 1, 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		μ moles/l	mg/100 ml	sec.		hr.
1. Butter	33.4	50	663 \pm 83	192 \pm 22	680 \pm 20	380 \pm 20	130 \pm 2	16.2 \pm 2.1	6.8 \pm 1.0
2. Arachis oil	94.8	13	720 \pm 50	78 \pm 9			160 \pm 7	9.4 \pm 2.5	18.3 \pm 1.7
3. Coconut oil	7.8	25	985 \pm 37	125 \pm 6	590 \pm 50	340 \pm 10	164 \pm 7	1.8 \pm 1.7	10.8 \pm 1.8
4. Horse tallow	82.7	25	234 \pm 29	18 \pm 1	420 \pm 10	420 \pm 10	134 \pm 6	1.8 \pm 1.3	16.6 \pm 1.7
5. Chicken tallow	79.3	28	459 \pm 40	80 \pm 8	460 \pm 30	390 \pm 10	141 \pm 4	3.7 \pm 0.9	16.7 \pm 1.4
6. Olive oil	84.4	23	1022 \pm 101	132 \pm 13	370 \pm 40	400 \pm 20	114 \pm 4	0.9 \pm 0.4	11.1 \pm 2.0
7. Bovine tallow	48.3	28	1016 \pm 100	186 \pm 19	460 \pm 20	480 \pm 40	165 \pm 6	0.8 \pm 0.4	9.3 \pm 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.

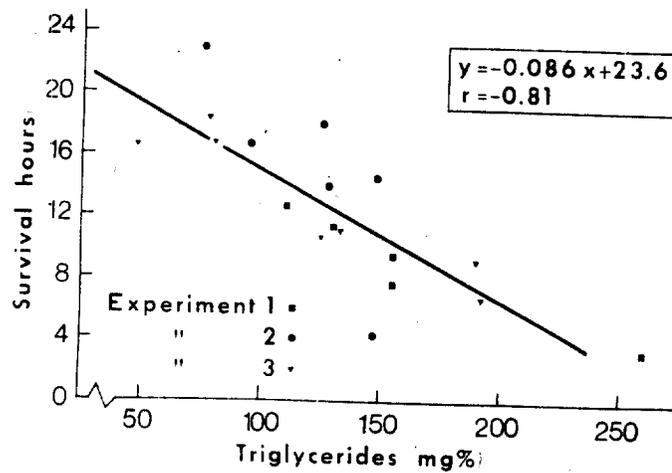


Fig. 2. The relationship, on a group basis, between the serum triglyceride level and the survival time in endotoxin-injected rats. The results are the pooled data of experiments 1, 2 and 3.

TABLE 5

Experiment 4: influence of dietary fats on lipemia and atherosclerosis

Results = Mean \pm S.E. Total duration of the experiment 18 weeks. Diet composition in addition to the fat (butter 38 per cent, other fats 32 per cent + 6 per cent water): casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight per cent).

Dietary Fat	No. of Autopsied Animals	Cholesterol		Triglycerides		Atherosclerosis (Scale 0-3)
		Week 10	Week 18	Week 10	Week 18	
		<i>mg/100 ml</i>		<i>mg/100 ml</i>		
Cacao butter.....	4	1136 \pm 27	2775 \pm 654	217 \pm 14	582 \pm 175	2.3
Butter.....	5	459 \pm 11	2001 \pm 696	124 \pm 10	413 \pm 160	1.7
Coconut oil.....	6	711 \pm 9	516 \pm 36	156 \pm 25	117 \pm 12	1.5
Olive oil.....	6	680 \pm 203	1621 \pm 342	188 \pm 56	309 \pm 70	0.3
Corn oil.....	6	301 \pm 12	383 \pm 34	57 \pm 3	76 \pm 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 noted⁹ 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 observed²³ that, after the liver, the tissues retaining the largest quantity of toxins and the most frequent site of thrombosis are the spleen and the lungs. However, at the dosage of endotoxin utilized in this experiment, occlusive thrombi are found almost exclusively in the liver; therefore, from a practical point of view, only this organ has been considered in evaluating the severity of the thrombotic process.

The results of experiment 1 in this work confirm our previous results^{12, 13} in that palmitic acid is much more hyperlipemic than stearic and caprylic acid, a finding that is also concordant with results obtained in man.^{14, 16} In contrast to this, our observations with myristic and lauric acids are markedly different from those reported in man.^{14, 16} Myristic acid induced a lipemia comparable to that induced by caprylic acid, and lauric acid was by far the least hyperlipemic of the five saturated fatty acids examined here. Of course, these results were obtained with purified free fatty acids and, as previously discussed,¹³ the digestibility of the corresponding triglyceride can differ markedly from that of the acid and could explain the discrepancy in the results obtained when fats are used. However, among the fats studied in this series, only coconut oil contained large amounts of lauric and myristic acids. The results of experiment 3 showed that even in large-scale experiments, the cholesterol was the same in animals fed butter as in those fed coconut oil, and the triglycerides were lower in the latter group. Therefore, under our conditions, lauric and myristic acids cannot be highly hyperlipemic. From acute experiments with coconut oil in the dog,²⁴ it was concluded that saturated fatty acids of 12 and 14 carbon atoms had the most powerful effect in raising serum cholesterol, a finding that could also be applied to humans.¹⁵

However, the reason for this is far from clear, inasmuch as it has been reported that coconut oil decreases cholesterolemia in hyperlipemic patients²⁵ 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 Aitiu-Mitiaro islanders²⁸ 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 thrombogenic¹³ than linoleic acid, and only slightly more atherogenic.^{12, 13} However, we also found that purified triolein was as thrombogenic as tristearin.¹³ Under the present conditions, the only interpretation that can be offered for the surprisingly low thrombogenicity of olive oil and mostly bovine tallow is that oleic acid, whether in its free or in its triglyceride form, is not thrombogenic. Another surprising result is the thrombogenicity of arachis oil despite its high content in oleic and linoleic acid. This, however, confirms exactly what we had previously reported¹¹ 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 clear relationship is obvious between various parameters and the severity of thrombosis, whereas in experiment 3, where a large number of various fats were used, none of the parameters examined could really be utilized for predicting the severity of thrombosis, a result similar to that already reported with various fatty acids.^{12, 13} Of course, large thromboses cannot be initiated by endotoxins in animals with a serum cholesterol level below 200 mg and with a clotting time longer than 180 sec. On the basis of a large scale experiment, however, all that can be said is that these parameters represent no more than risk factors and by no means serve to predict closely the thrombotic tendency. From our experience with the addition of free fatty acids to the diet, these results are not surprising, because we have observed that the thrombogenicity of the different fatty acids does not necessarily parallel their effect on serum cholesterol or triglycerides. If, however, as suggested by these experiments, the thrombotic tendency is mostly due to the effect of some fatty acids on blood platelets, the parameters examined, except to some extent the clotting time, do not indicate this. Nevertheless, although the plasma clotting time as performed under our conditions is a highly constant and reliable determination, it is much more affected by the environmental conditions of the animal than the thrombotic phenomenon itself and, therefore, does not appear to constitute the ideal test.

Under the present conditions, it seems clear that neither the origin of a fat nor its degree of saturation governs its lipemic, thrombogenic or atherogenic effects. The type of fatty acids it contains is probably responsible for the above-mentioned effects; but due to the number of fatty acids involved and the various interactions observed to occur with such dietary feeding,³⁰ many more experiments will need to be done before we can completely predict the lipemic and thrombogenic effects of certain fats or fat mixtures. Also, with so many data to deal with, the help of a computer will probably be necessary, as other investigators have realized under similar conditions.³⁰ Another source of error is that, except for four samples, the composition in fatty acids of the fats used here has not been determined and could be slightly different from those reported in table 3.

As regards the mortality rate and the survival time, in almost none of the present or previous experiments did we find it to be related to the severity of the occlusive thrombosis. At least on a group basis, the survival time can be correlated with the degree of lipemia, as previously observed,¹⁰ and the mortality is probably attributable to shock. In rabbits treated by injection with bacterial endotoxin, it was also reported that the mortality rate was related to the triglyceride level.³¹

From a practical point of view, it seems essential to evaluate to what extent the present experimental model in the rat yields results that are comparable to those obtained in man and also the degree to which it could help to clarify the pathogenesis of large thrombosis and coronary disease in man. Under our conditions, after cacao butter, which has also been found to be highly atherogenic in the rabbit,^{20, 32} the most thrombogenic fat is butter and, therefore, probably the other dairy products also. Dairy products represent a large portion of the dietary fat of the highly civilized countries with a high rate of coronary disease. Coconut oil, although able to induce a degree of cholesterolemia similar to that of butter, does not seem to be highly thrombogenic in man²⁸ or under our experimental conditions. Olive oil, like oleic acid a non-thrombogenic 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 *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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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 (Cere-lose). 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%. Anderson³ 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 Sunde⁷ 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 Mattson⁸ 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 Reiser⁹ 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 Deuel¹⁰ and Langworthy¹¹ postulated that digestibility is inversely proportional to and dependent on melting point, while Hoagland and Snider¹² and Mattil¹³ 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 coworkers¹¹ 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 %
<i>Edible fats</i>			
Corn oil	122	3950	92.9
Degummed soybean oil	133	4210	98.8
Lard	65	3980	93.4
Hydrogenated fat	69	3250	76.2
<i>Inedible fats</i>			
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
<i>Fatty acids</i>			
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
<i>Miscellaneous</i>			
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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EXPERIMENTS ON THE GROWTH AND LONGEVITY
OF THE WHITE MOUSE

1. THE INFLUENCE OF INJECTIONS OF THORIUM OLEATE IN OLEIC ACID,
AND OF OLEIC ACID ALONE, ON GROWTH AND LONGEVITY

by

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These experiments were planned and begun in 1929 by the late Professor Brailsford Robertson, and since his death (in January, 1930) have been carried on by his colleagues at the Laboratory of the Division of Animal Nutrition.

EXPERIMENTAL PROCEDURE.

The animals used were drawn from the stock of albino mice previously described by Robertson (1925). They were removed to the experimental cages at five weeks of age, a few days after being weaned. Control and experimental animals were chosen alternately from the same litters. Obviously weak animals and those which were the only members of their litters were rejected. Litters of more than five were reduced to five at birth.

The mice were confined in cages of metal and Vita glass, with floors of perforated zinc. Each cage contained a cubical earthenware nest, in which the animals slept on sawdust bedding. The cages were carefully cleaned each week. Six mice, all of one sex, shared each cage.

The animal room was lit from above, and from the east with Vita glass windows, and was maintained at 70° F. in the winter. Despite the use of ventilating fans, the temperature sometimes rose above 90° F. during summer.

The standard mixed diet, which was fed to all the mice *ad lib.*, consisted of yellow maize which had been dipped in boiling water, and rice boiled in milk, to which a small quantity of NaCl had been added.

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In addition each animal received 1 c.c. of a mixture of equal parts of whole egg and fresh milk on six days a week. Any dietary supplements were mixed with the egg and milk, which was given to the animals before the other food, to ensure its being eaten. It was not practicable to give each mouse its share of egg and milk separately; 6 c.c. was shared between six animals. Water was given by the drop tube method.

A single experimental unit of 144 animals was used, divided into four equal sub-groups.

Thirty-six male mice received injections of an emulsion of thorium oleate in oleic acid from 5 weeks to 400 days of age. These were the "experimental males." The "control males" received injections of an oleic acid emulsion containing no thorium. The "experimental females" received injections of the thorium oleate emulsion, but, unlike the males, their diet was supplemented by the daily² addition of 25 mgs. of vegetable nucleic acid (British drug houses) throughout life. The "control females" were treated exactly like the "control males."

The emulsion of thorium oleate was prepared as follows:

20 c.c. of oleic acid were placed in a wet Erlenmeyer flask and 4 gms. of finely-ground thorium oleate (B.D.H.) added to it. The mixture was then shaken and gently heated over a small flame until the thorium oleate was completely dissolved. To it was then added 400 c.c. of boiling .35% NaOH, and the whole violently shaken for some minutes, and allowed to cool. An excellent emulsion was produced. Owing to the variable composition of thorium oleate, it was necessary to standardize the emulsion to determine the dosage of thorium administered with each injection. This was done by taking suitable aliquots of the emulsion and evaporating to dryness in a platinum dish. Sufficient sulphuric acid to decompose the oleate present was then added, and the dish and its contents were heated to fuming. The remainder of the procedure for estimating the thorium present was by precipitation as iodate, in the manner detailed later. The thorium oleate emulsion usually contained approximately 2 mgs. thorium dioxide per c.c., actually .0018 gms. ThO₂ per c.c.).

The oleic acid emulsion was prepared in the same way as the thorium oleate emulsion, except that no thorium oleate was added.

Prior to the injections, the required amounts of the emulsions were sterilized.

The material was injected subcutaneously, usually in the back. Until the mice reached 20 grms. in weight each one was given a weekly injection of 0.25 c.c. of one or other emulsion. Afterwards the amount was increased to 0.5 c.c.

As both control and experimental animals sometimes developed sores at the sites of the injections, after 210 days of age the injections were given fortnightly. The condition of the mice greatly improved, the development of sores being much less frequent. Nevertheless, although no injections were made after 400 days of age, there were animals whose sores did not heal, even though they lived to upwards of 700 days. This rarely happened among the control animals. The animals were weighed each week till they were 210 days old, and fortnightly thereafter.

² Each day except Sunday.

PRESENTATION OF RESULTS.

The figures show the average weights of the animals from five weeks of age till the death of the last animal. The "probable error" of the mean weight at each age $(0.6745 \times \text{standard deviation})$ has been plotted on each side of the

I n

mean, till "n," the number of animals, was reduced to five. This method, by which each growth curve appears as a ribbon instead of a line, was previously used by Robertson (1925).

In the mortality statistics, a few deaths which could be reasonably attributed to accident, have been excluded. Of these there were only 9 among 425 animals.

There was no epidemic infection among the mice.

In each group the percentage of the original number of animals surviving has been calculated at 50-day intervals, and shown underneath the growth curve.

The mean durations of life are represented by circles of which the centres are situated at the observed average age of death, and the diameters are twice the probable errors of the average.

The injections of oleic acid do not seem to have affected the growth and weight of the mice, for the control animals, both male and female, showed growth curves comparing favourably with those of the control mice in other experiments carried out at the same time. They do, however, appear to have reduced the expectation of life of the animals. This effect can be gauged by comparing the mean life duration of the control animals in this experiment with that of the control animals in two experiments to be described in the succeeding paper in this Journal. For the male animals the life durations were 661 days in this experiment, and 772 and 775 days in the other experiments; for the female animals 702 days as compared with 759 and 801 days.

The injection of thorium oleate depressed the growth of the experimental animals, and the mean maximum weight obtained by them was less than when only oleic acid was injected.

The mortality statistics also point to an injurious effect of the thorium, *per se*. The mean life duration of the experimental male animals was 44 days less than that of the controls, a difference which may or may not be significant. In the case of the females the difference was 71 days (p.e. 24), and was certainly significant.

The female mice appeared to be slightly more sensitive to the action of thorium than the males. It is doubtful whether this apparent increase in sensitivity was due to the vegetable nucleic acid in their diet.

The Retention of Thorium in the Bodies of the Mice, and its Distribution in Different Organs.

The distribution of the injected thorium among the several organs and tissues of the mice was qualitatively investigated in some instances by ashing the tissues and making photo-radiographic tests by exposing a photographic plate, enclosed

in a light-proof box, to the radiations from the thorium in the ash. Exposures ranged from 14 to 30 days. By this means the presence of traces of thorium in most of the tissues was confirmed, and the variations in thorium concentration in the several organs were also made apparent.

By reason of the fact that the radiations are absorbed proportionately to the amount of ash present, and its density, this method cannot be considered strictly quantitative, but the results show that the thorium tends to accumulate preferentially in the bones, skin, and kidneys, in the order stated, under the conditions of its administration in this experiment. It was found that thorium was relatively absent from the blood, brain, liver, heart, spleen, submaxillary gland, intestine, lungs, and fat. If the dry weights of the above-mentioned organs and tissues are taken into consideration it is at once apparent from the photographic results that the bulk of the retained thorium was present in the bones. In view of the great insolubility of thorium phosphate, in which form the thorium presumably occurred in the bones, this result might well have been expected on *a priori* grounds.

Quantitative determinations of the amount of thorium retained, compared with the amount administered, were made on seven mice only. The analytical procedure was as follows:

The entire mouse was placed in a platinum dish, and dried in an air oven at 100°C., after which it was completely ashed in an electric muffle furnace. The ash was moistened with strong sulphuric acid, and heated to fuming to render soluble any oxide or phosphate of thorium. When cool, the sample was dissolved in nitric acid of such a strength as to preclude the precipitation of calcium sulphate or phosphate, and the thorium was then precipitated by cautious addition of a saturated solution of potassium iodate. After standing 24 hours, the precipitated thorium iodate was filtered off and washed with weak nitric acid containing potassium iodate. It was then dissolved, on the filter, in hot hydrochloric acid, of 6 N. strength, and washed through into a small beaker. The solution was then evaporated on a water-bath, to reduce the iodate and transform the thorium to chloride. The residue was then taken up in a little water with a trace only of weak hydrochloric acid, and the thorium precipitated as peroxy-hydroxide by addition of pure hydrogen peroxide solution, and subsequent warming of the solution to 70°C. The white flocculent precipitate was filtered off, washed, dried, and carefully ignited with the filter in a platinum crucible to constant weight.

In this way the following results for retained thorium dioxide were obtained:

Mouse number.	Sex.	Calculated thorium dioxide injected. (gms.).	Thorium dioxide recovered. (gms.).	% Dosage ThO ₂ retained in tissues.	Age at death (days).	Days since last injection of Thorium.
G.i.15	M.	0.032	0.0150	46.9	440	42
G.i.17	M.	0.025	0.0149	59.6	334	12
H.k.13	F.	0.017	0.0060	35.3	288	1
H.s.28	F.	0.028	0.0100	35.7	382	6
H.b.1	F.	0.038	0.0060	15.8	538	132
G.e.12	M.	0.031	0.0062	20.0	383	6
G.a.5	M.	0.031	0.0080	25.8	399	8

From the above table it appears that the amount of thorium retained by the animal bore no fixed relation to the amount injected, even when the time interval since the last administration of thorium is taken into consideration. The amounts of thorium excreted with the urine and faeces respectively were not determined

in the present investigation. However, the results obtained by v. Hevesy and Wagner (1930), in a similar experiment, in which thorium nitrate, containing traces of uranium X, was subcutaneously injected into mice, and subsequently determined electrometrically, showed that, of the total amount of thorium excreted 64% was passed in the urine and 36% in the faeces. In view of this fact the possibility of the deleterious action of the thorium, irrespective of its radiations, on the kidney tissue should be taken into account, not only as a probable factor in determining the duration of life of the animals, but also as leading to a restricted excretion of thorium by this channel, and therefore possibly explaining the irregular retention of thorium shown in the above results.

SUMMARY.

The injection of an emulsion of 1% thorium oleate in oleic acid into male and female white mice at short intervals up to 400 days of age depressed their growth below that of the controls, which received injections of oleic acid only. There is reason to believe that the oleic acid injections had no influence on growth.

Both oleic acid and thorium oleate injections shortened the life-span of the animals. The effect of the thorium was more marked than that of the oleic acid.

Analysis of some of the mice after death showed that not all the thorium had been excreted. The amount retained varied from 16% to 60% of that injected, and was present principally in the bones, the skin, and the kidneys.

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TABLE I.

Growth of male white mice receiving injections of oleic acid, 1929-1932.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
36	5	9.40	.16	34	25	26.16	.20
36	6	11.09	.21	24	26	26.36	.26
36	7	12.70	.24	34	27	26.70	.26
36	8	13.34	.22	34	28	26.91	.25
36	9	14.43	.31	34	29	27.10	.29
36	10	15.73	.26	34	30	27.09	.27
36	11	17.33	.25	33	32	27.53	.30
36	12	18.66	.24	33	34	27.56	.32
36	13	19.63	.22	33	36	28.20	.36
36	14	20.62	.22	33	38	28.38	.36
35	15	21.25	.21	33	40	29.00	.37
35	16	21.95	.24	33	42	29.68	.38
35	17	22.58	.23	33	44	29.85	.41
35	18	23.08	.23	33	46	30.42	.44
35	19	23.70	.25	32	48	30.88	.48
35	20	24.00	.24	32	50	31.64	.56
35	21	24.35	.23	32	52	31.72	.57
35	22	25.10	.23	31	54	32.22	.62
34	23	25.52	.26	31	56	32.42	.69
34	24	25.88	.26	31	58	32.65	.74

TABLE 1 (continued).

Growth of male white mice receiving injections of oleic acid, 1929-1932.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
30	60	33.37	.79	13	104	29.85	.64
28	62	34.20	.78	11	106	29.23	.85
28	64	34.21	.81	9	108	29.94	.57
28	66	33.77	.91	8	110	29.44	.42
27	68	33.74	.97	7	112	29.43	.48
27	70	33.78	1.05	7	114	29.07	.49
27	72	33.43	1.01	6	116	28.58	.57
26	74	33.67	1.03	6	118	28.42	.61
26	76	33.37	1.04	6	120	27.33	.64
26	78	31.85	.99	5	122	27.80	.54
24	80	32.88	.95	5	124	27.40	.64
23	82	32.93	.66	3	126	27.33	—
21	84	31.79	.71	3	128	26.50	—
18	86	31.75	.75	3	130	26.33	—
17	88	32.00	.64	3	132	26.50	—
17	90	31.65	.65	3	134	25.33	—
17	92	31.41	.65	2	136	23.25	—
16	94	31.69	.59	1	138	24.50	—
16	96	31.06	.56	1	140	24.50	—
14	98	31.07	.61	1	142	24.50	—
14	100	30.32	.57	1	144	23.50	—
13	102	30.23	.63	1	146	23.00	—

TABLE 2.

Growth of male white mice receiving injections of thorium oleate in oleic acid 1929-1932.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
36	5	9.21	.17	36	30	26.56	.30
36	6	11.08	.21	36	32	27.01	.28
36	7	12.72	.24	35	34	27.57	.32
36	8	12.93	.31	35	36	27.97	.30
36	9	13.92	.30	35	38	28.30	.34
36	10	15.11	.31	35	40	28.74	.33
36	11	16.67	.32	35	42	29.01	.33
36	12	18.15	.27	35	44	29.44	.34
36	13	18.81	.24	34	46	29.56	.39
36	14	19.69	.26	33	48	29.59	.35
36	15	20.25	.25	33	50	29.95	.40
36	16	20.81	.24	33	52	29.88	.40
36	17	21.33	.24	31	54	30.79	.47
36	18	21.75	.25	30	56	30.80	.52
36	19	22.21	.26	28	58	31.73	.55
36	20	22.74	.27	28	60	31.68	.61
36	21	23.33	.27	26	62	32.40	.65
36	22	23.85	.28	26	64	32.40	.65
36	23	24.46	.26	26	66	32.79	.70
36	24	24.60	.27	25	68	32.92	.74
36	25	25.08	.27	23	70	32.80	.82
36	26	25.40	.26	23	72	32.37	.73
36	27	25.78	.27	23	74	32.30	.69
36	28	26.06	.29	23	76	32.28	.71
36	29	26.42	.29	23	78	32.09	.70

TABLE 2 (continued).

Growth of male white mice receiving injections of thorium oleate in oleic acid, 1929-1932.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
23	80	31.28	.73	5	116	27.20	.57
22	82	30.48	.73	4	118	29.12	—
21	84	30.83	.59	3	120	27.67	—
20	86	31.13	.65	2	122	27.75	—
19	88	30.47	.61	2	124	27.50	—
19	90	29.97	.55	2	126	28.25	—
19	92	29.32	.58	2	128	27.50	—
18	94	29.36	.61	2	130	27.00	—
17	96	29.59	.66	2	132	26.50	—
15	98	29.67	.71	1	134	28.50	—
14	100	28.86	.64	1	136	27.00	—
12	102	29.67	.69	1	138	27.00	—
11	104	29.23	.56	1	140	27.00	—
10	106	28.55	.61	1	142	27.00	—
10	108	27.20	.72	1	144	25.00	—
7	110	29.07	.67	1	146	25.50	—
6	112	28.83	.79	1	148	25.00	—
6	114	28.75	.62	1	150	24.50	—

TABLE 3.

Growth of female white mice receiving injections of oleic acid, 1929-1931.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
36	5	9.82	.17	35	40	26.63	.30
36	6	11.99	.21	35	42	26.81	.30
36	7	12.88	.19	34	44	27.56	.33
36	8	13.85	.21	34	46	28.09	.38
36	9	14.54	.23	34	48	28.68	.42
36	10	15.75	.21	34	50	28.53	.46
36	11	16.61	.23	33	52	29.24	.51
36	12	17.63	.23	32	54	30.22	.53
36	13	18.26	.19	32	56	30.55	.54
35	14	18.87	.19	32	58	30.97	.55
35	15	19.41	.20	32	60	31.13	.55
35	16	20.61	.20	32	62	31.14	.52
35	17	20.54	.21	32	64	31.02	.50
35	18	20.91	.20	32	66	31.20	.49
35	19	21.26	.21	31	68	30.94	.53
35	20	21.64	.22	31	70	30.71	.60
35	21	22.00	.22	30	72	30.83	.53
35	22	22.24	.24	30	74	31.30	.48
35	23	22.73	.27	30	76	31.27	.54
35	24	22.97	.26	30	78	31.18	.51
35	25	23.10	.25	29	80	31.34	.48
35	26	23.43	.24	29	82	30.95	.50
35	27	23.83	.26	29	84	30.59	.49
35	28	23.91	.25	29	86	30.21	.50
35	29	24.11	.25	29	88	29.57	.49
35	30	24.31	.26	29	90	29.28	.47
35	32	24.89	.29	28	92	29.14	.53
35	34	25.00	.26	28	94	28.34	.50
35	36	25.80	.28	26	96	28.27	.51
35	38	26.10	.31	25	98	27.66	.63

TABLE 3 (continued).

Growth of female white mice receiving injections of oleic acid, 1929-1931.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
22	100	28.48	.59	4	120	26.12	—
20	102	27.70	.68	4	122	24.75	—
16	104	28.06	.65	3	124	24.33	—
15	106	26.97	.56	2	126	20.75	—
13	108	27.04	.56	2	128	20.50	—
10	110	26.25	.76	1	130	23.00	—
9	112	26.61	.62	1	132	25.50	—
8	114	25.81	.53	1	134	24.00	—
8	116	24.94	.68	1	136	23.50	—
5	118	26.20	.39				

TABLE 4.

Growth of female white mice receiving injections of thorium oleate in oleic acid, 1929-1931.

Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.	Number of animals.	Age in weeks.	Mean weight gms.	Probable error of mean.
36	5	9.33	.14	34	54	28.90	.46
36	6	11.17	.18	33	56	29.06	.46
36	7	12.29	.22	32	58	29.52	.47
36	8	12.81	.22	31	60	29.71	.46
36	9	13.51	.22	31	62	29.63	.51
36	10	13.89	.20	31	64	29.31	.52
36	11	15.18	.23	30	66	29.92	.58
36	12	16.24	.21	30	68	29.75	.54
36	13	17.08	.20	29	70	29.60	.55
36	14	17.79	.21	29	72	29.12	.55
36	15	18.43	.21	28	74	29.02	.58
36	16	18.69	.21	27	76	28.52	.73
36	17	19.24	.22	25	78	29.26	.66
36	18	19.60	.23	24	80	29.46	.70
36	19	19.90	.23	22	82	29.91	.57
36	20	20.21	.26	22	84	29.07	.59
36	21	20.42	.28	21	86	28.64	.55
36	22	20.90	.26	21	88	27.98	.66
36	23	21.26	.30	19	90	28.13	.64
36	24	21.71	.31	19	92	27.37	.59
36	25	21.83	.33	19	94	27.00	.56
36	26	22.22	.31	16	96	27.88	.60
36	27	22.63	.30	16	98	27.34	.54
36	28	22.79	.28	14	100	26.79	.64
36	29	23.01	.28	11	102	26.41	.71
36	30	23.36	.27	9	104	25.61	.86
36	32	23.67	.30	7	106	25.86	.97
36	34	24.08	.30	7	108	24.50	.82
36	36	24.78	.30	6	110	25.42	.70
36	38	25.07	.33	6	112	25.10	.70
36	40	25.51	.33	6	114	25.75	.36
35	42	25.86	.33	6	116	24.00	.41
35	44	26.63	.35	5	118	23.10	.72
35	46	26.87	.36	2	120	23.75	—
35	48	27.37	.37	2	122	23.50	—
35	50	27.57	.43	1	124	18.50	—
34	52	28.43	.38				

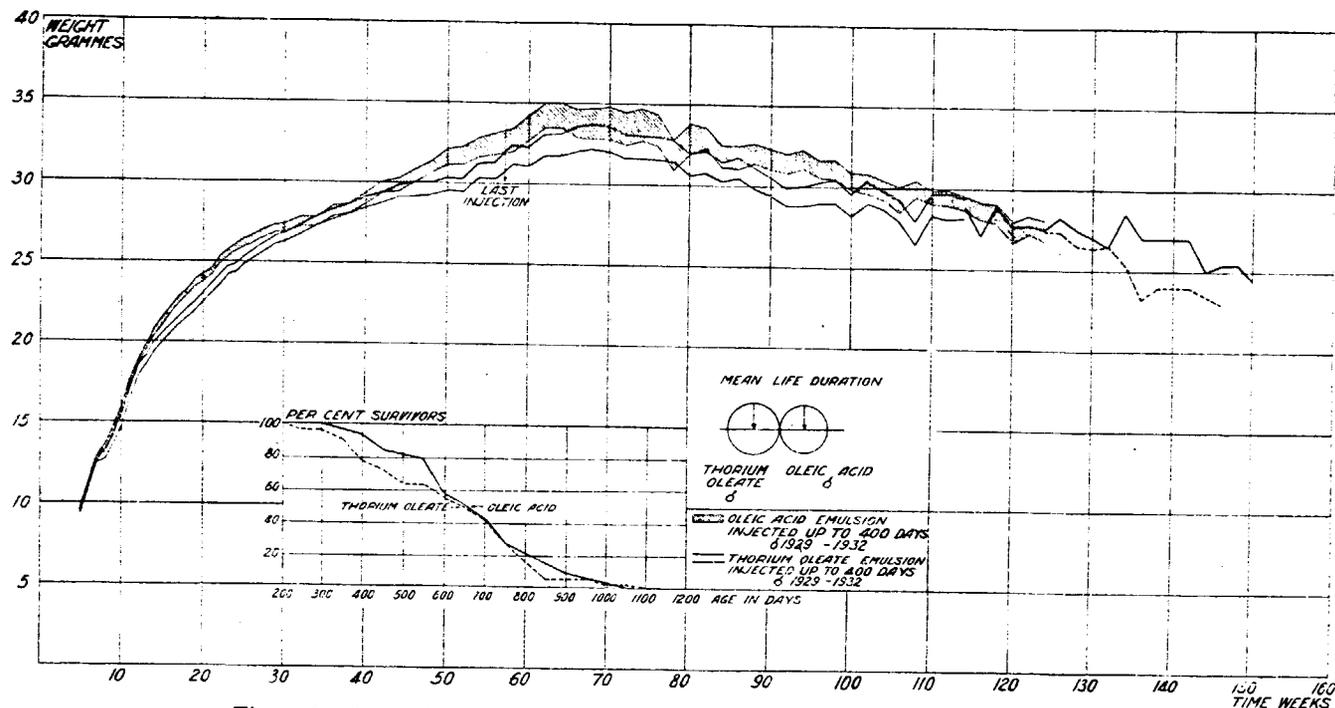


Figure 1. Comparison of the growth and longevity of male mice receiving injections of an emulsion of oleic acid, and male mice receiving injections of an emulsion of 1% thorium oleate in oleic acid.

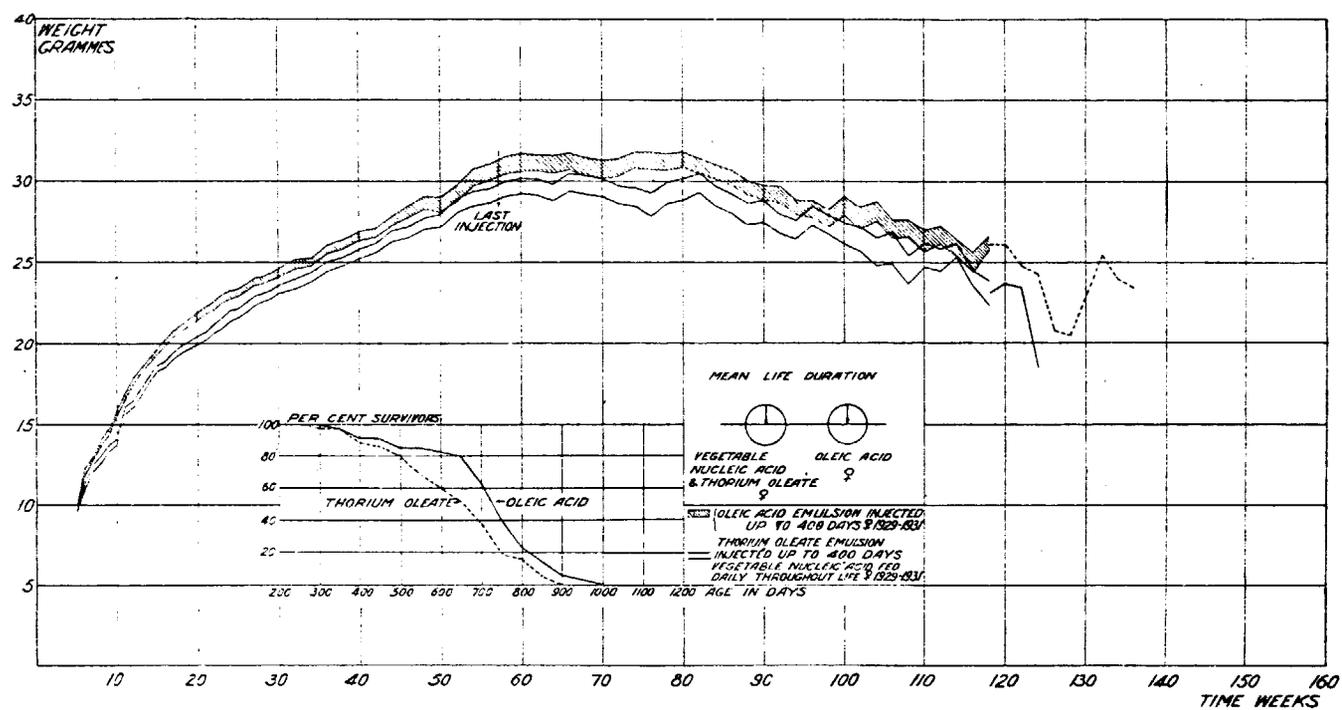


Figure 2. Comparison of the growth and longevity of female mice receiving injections of an emulsion of oleic acid, and female mice receiving injections of an emulsion of 1% thorium oleate in oleic acid, the latter with a dietary supplement of 25 mgs. of vegetable nucleic acid daily.

CHROMIUM CARCINOGENESIS: CALCIUM CHROMATE AS A
POTENT CARCINOGEN FOR THE SUBCUTANEOUS TISSUES
OF THE RAT

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OVER a period of 15 years, we have investigated (at the request of the chromates-producing industry in Great Britain) the carcinogenicity of several different chromium-containing compounds by the technique of single or repeated intramuscular injection in rats and/or mice. The results of these tests were invariably negative until, at the independent suggestions of Dr. Sidney Laskin and Dr. Lesley Bidstrup, calcium chromate was examined in the same way.

The results of this experiment are now reported. Although a strikingly high yield of tumours was obtained, the present findings do no more than confirm those of Hueper and Payne (1959). But independent corroboration of their assertions in respect of calcium chromate seemed especially necessary in view of our inability to induce injection-site tumours with chromite ore roast, chromite-roast residues or chromic chromate—all of which Hueper and Payne reported as carcinogenic on intramuscular (and also intrapleural) injection in rats (Hueper, 1958; Hueper and Payne, 1959).

The evidence for an association between exposure to chromates and lung cancer in man, critically reviewed by Gruschko (1961), provides the rationale for examining chromium-containing compounds for carcinogenicity. If certain industrial processes could be pin-pointed as especially hazardous, it would be possible to direct protective measures, currently being taken in all sectors, to those parts of the process which appear to be more likely to be associated with the risk of lung cancer. This might have the advantage to the industry of reducing the cost of ensuring that the risk is eliminated. Calcium chromate is formed in varying amounts when chromite ore is heated with limestone and sodium or potassium carbonate—the first step in processing the crude ore—and this would appear to be an important stage where potential carcinogens might be encountered.

The present findings should be considered alongside those of Laskin and his colleagues who, at the meeting of the American Industrial Health Association in St. Louis in May 1968, reported the induction of squamous carcinomas of the lung in 6, and adenocarcinomas of the lung in 2, out of 100 rats exposed to calcium chromate in the form of a pellet attached to the bronchial mucosa.

MATERIALS AND METHODS

Experimental animals.—Forty male CB stock rats, aged 5 to 6 weeks at the beginning of the experiment, were used. They were kept in metal cages, 5 in each, and fed on cubed diet No. 86 (Messrs Dixon Ltd., Ware, Herts) and water *ad libitum*.

Calcium chromate.—Calcium chromate was obtained from British Drug Houses, Ltd.

Conduct of experiment

The rats were divided into a test group of 24 animals and a control group of 16 animals. The *test group* received 20 once-weekly injections of calcium chromate suspended in 0.2 ml. arachis oil, given intramuscularly into the right flank. For the first 2 injections, the dose of calcium chromate was 5 mg. but signs of severe inflammation developed at the site of injection and the dose was reduced to 0.5 mg. for the remaining 18 weeks. The test animals thus received 19 mg. calcium chromate over a span of 20 weeks. The *control group* received 20 once-weekly injections of 0.2 ml. arachis oil, given intramuscularly into the right flank.

The rats were observed daily and examined for changes at the injection site. Animals were killed when they developed tumours or at the termination of the experiment at 63 weeks. Full post mortem examinations were carried out and tumours and tissues showing macroscopic abnormalities were removed and fixed in Bouin's solution. Paraffin sections were prepared at 5μ and stained with haematoxylin and eosin.

RESULTS

The results are shown in Table I. No systemic toxic effects attributable to calcium chromate were observed. During the period when injections were given, the injection sites became swollen and appeared tender but no ulceration of the overlying skin developed. The local swelling later subsided but there remained slight palpable thickening of the subcutaneous tissues at the injection site.

Tumours developed at the injection site in 18 of the 24 test animals (75%)—Table I. The first lesion appeared 203 days after the beginning of the experiment

TABLE I.—*Carcinogenesis in Rats Injected Subcutaneously with Calcium Chromate*

	Days after the beginning of experiment						
	150	200	250	300	350	400	440
<i>Test group</i>							
Survivors	24	24	24	22	17	9	0
Cumulative total of tumour-bearing rats	0	0	3	6	11	16	18
Spindle cell sarcomas	0	0	2	4	8	11	11
Pleomorphic sarcomas	0	0	1	2	3	5	7
<i>Control group</i>							
Survivors	15	14	13	12	12	9	0
Total of tumour-bearing rats	0	0	0	0	0	0	0

and the overall mean period of induction was 323 days. Once palpable, the tumours grew rapidly; all tumour-bearing rats were killed before their lesions began to ulcerate and the interval between the first detection of a tumour and the time when the rat had to be killed was, on average, 29 days. The neoplasms induced by calcium chromate were either spindle cell or pleomorphic cell sarcomata (Fig. 1 and 2). Some of the spindle cell lesions showed quite marked collagen formation. The pleomorphic tumours contained virtually no collagen fibres and were composed of bizarre binucleate and multinucleate cells, many of them with

abnormal mitotic figures. No deposits of chromate were observed in or around the tumours. The sarcomas showed wide local invasion of the subcutaneous tissues, with extension into the muscles of the body wall, but no metastatic deposits were found.

The injection sites in the 6 test rats which did not develop local tumours, and in the 16 control animals injected with arachis oil alone, showed no specific features. There was no epidermal ulceration. The dermis contained increased amounts of fibrous tissue, infiltrates of macrophages and chronic inflammatory cells, and dilated lymphatic vessels. These changes were more prominent in rats injected with calcium chromate than in rats which were given arachis oil only.

No distant tumours developed in any rats in the test or control groups. Some animals from both groups showed bronchiectasis and cystic nephritis (sometimes accompanied by hydronephrosis) but there was no difference in the incidence, extent, or severity of these lesions between the test and control groups.

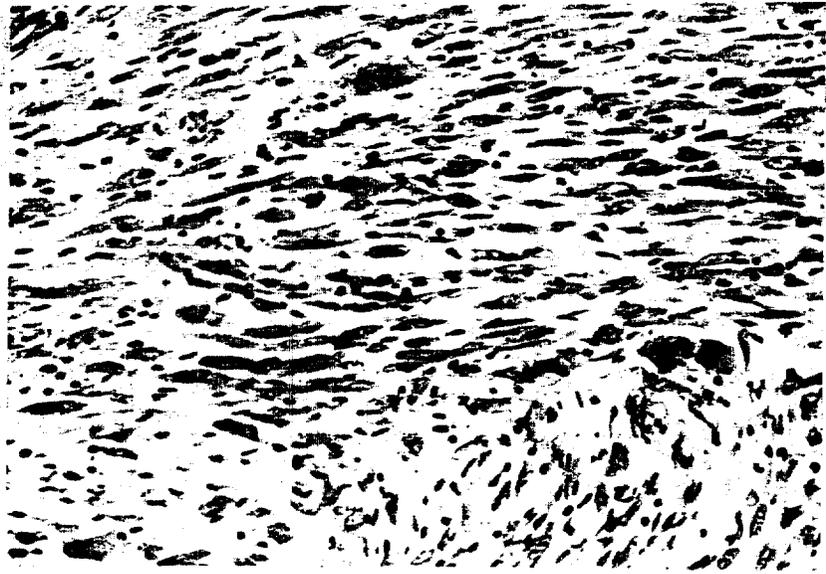
DISCUSSION

In 1827, Cumin described ulcers and sloughs "of a peculiarly penetrating character" on the hands and forearms of Scottish workers who handled "bi-chromate of potass". Similar cutaneous lesions were observed by later writers and perforating septal ulcers of the nose were reported in chromate workers by Delpech and Hillairet in 1869 (see Gafafer, 1952). Neither of these lesions was pre-neoplastic, no damage to other tissues following exposure to chromium-containing compounds was noted, and it was not until the 1930's that an association between exposure to chromates and the development of malignant disease—lung cancer—was suspected. German investigators (see reviews by Machle and Gregorius, 1948; Baetjer, 1950; Gafafer, 1952) were the first to recognise this association but their data were inadequate to calculate the risk involved: furthermore, the diagnosis of lung cancer was not always firmly established and some patients had been exposed to other known carcinogens. Machle and Gregorius (1948) conducted a survey of chromate workers in the United States and reported an exceptionally high risk of lung cancer in men engaged in the production of chromates and, to a lesser extent, of chrome pigments. No increase in cancer of other sites was found. Despite certain criticisms of the design of Machle and Gregorius's study, Baetjer (1950) confirmed their final conclusion and showed that lung cancer was not only more common but also occurred earlier in the chromate workers, the highest incidence being found in the 40-49 age group. In the United Kingdom, Bidstrup and Case (1956) also reported an increased incidence of mortality for cancer of the lung in the chromate-producing industry and noted a tendency for death due to bronchogenic carcinoma to occur disproportionately early. Important non-occupational factors such as the smoking habits and social class of the workers, and diagnostic bias, were examined and considered to be

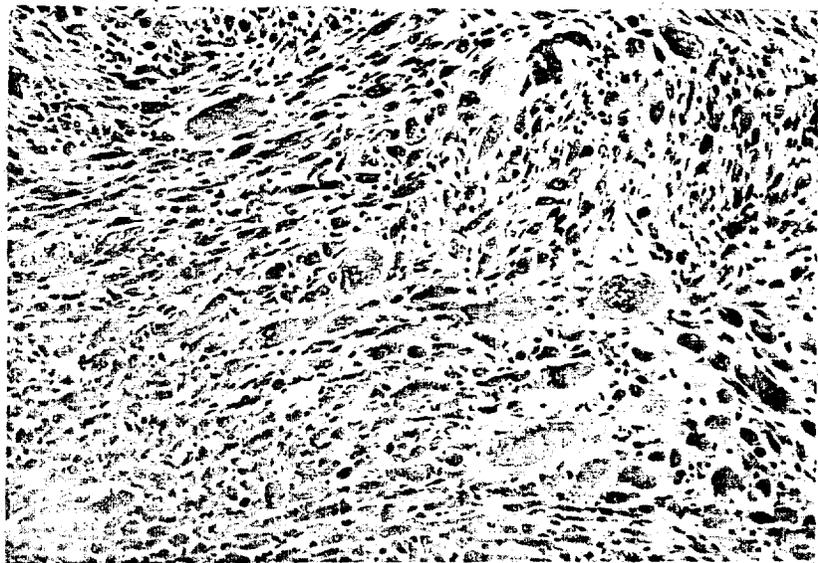
EXPLANATION OF PLATE

FIG. 1.—Spindle cell sarcoma arising at injection site of rat treated with calcium chromate. Tumour palpable at 273 days; rat killed at 300 days. H. and E. \times 230.

FIG. 2.—Anaplastic sarcoma arising at injection site of rat treated with calcium chromate. Tumour palpable at 314 days; rat killed at 339 days. H. and E. \times 180.



1



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Roe and Carter.

inadequate to account for the increased incidence of lung cancer. There is, therefore, abundant evidence that bronchogenic carcinoma is an occupational hazard in the chromate-producing industry.

Until recently, animal experiments have failed to provide a clear indication of the identity of the compound or compounds which increase the risk of cancer development in chromate workers. Hueper (1955) reported essentially negative results in a series of investigations in which various species were exposed to powdered chromium ore or to pure powdered metallic chromium, administered by various routes. In 1958, Hueper reported a small number of malignant tumours developing in rats after intramuscular or intrapleural administration of chromite ore roast. He stressed that the yield of neoplasms was small and that chromite ore contained other putative carcinogens as well as chromium; but in an addendum to this paper, he noted that calcium chromate implanted intramuscularly in rats produced local sarcomas. This observation was extended in a later paper (Hueper and Payne, 1959), in which it was shown that intramuscular implantation of calcium chromate or chromium trioxide in rats was followed by local tumours in 60-79% of animals. Calcium chromate was subsequently found to induce injection-site sarcomas in mice (Payne, 1960) but attempts to produce pulmonary tumours in rats by intratracheal administration of calcium chromate were unsuccessful (Hueper and Payne, 1962).

The present findings constitute a complete and independent confirmation of those of Hueper and Payne (1959) in respect of the potent carcinogenicity of calcium chromate for the subcutaneous tissues of the rat. As such, they may serve as a pointer to those concerned with the lung cancer hazard in the chromate-producing industry. The induction of pulmonary cancers in rats by the intrabronchial implantation of pellets containing calcium chromate or chromic chromate by Laskin and his colleagues (see introduction) may serve the same purpose.

SUMMARY

Twenty-four young male CB stock rats received 20 once-weekly injections of calcium chromate, suspended in 0.2 ml. arachis oil, given intramuscularly into the right flank; each rat received 19 mg. calcium chromate. Sixteen similar control rats received 20 once-weekly intramuscular injections of 0.2 ml. arachis oil alone. The experiment was terminated after 63 weeks when the surviving animals were killed.

Spindle cell and pleomorphic cell sarcomas developed at the site of injection in 18 of the 24 test rats (75%). The first tumour appeared 203 days after the beginning of the experiment and the mean period of induction was 323 days. The tumours were locally invasive but did not metastasise. No neoplasms were found at other sites and no injection-site tumours developed in control rats which received arachis oil alone.

These observations confirm the previous account by Hueper and Payne (1959) and indicate that calcium chromate is a potent carcinogen for the subcutaneous tissues of the rat. The present findings, particularly when taken in conjunction with the recent report by Laskin and his colleagues of lung cancers induced in rats with intrabronchial pellets of calcium chromate, indicate that this compound may be at least one of the carcinogens responsible for the well-documented cancer-hazard associated with the chromate-producing industry.

We thank Dr. Sidney Laskin and his colleagues for giving us advance information of the result of their experimental induction of pulmonary cancers in rats by the intrabronchial implantation of pellets of calcium chromate. We also acknowledge the advice of Dr. Lester Bidstrup, Professor R. A. M. Case and Dr. C. E. Dukes. We are grateful to Mr. B. C. V. Mitchley, Miss Norma Heathcote and Mr. George Munro for technical assistance and to Mr. K. G. Moreman and his staff for the photomicrographs.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research; Royal Cancer Hospital); from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant from the National Cancer Institute, U.S. Public Health Service.

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THE EFFECT OF THERMAL TREATMENT AND HYDROGENATION
ON THE ABSORPTION OF A FEW VEGETABLE OILS

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(Received for publication, January 1, 1944)

The comparative rates of absorption of different fats from the intestinal loops of rats has been reported by Steenbock *et al* (1). They found that certain fats are more rapidly absorbed than others and have also observed the effect thereon of many different substances. The absorption of different oils which have been thermally treated have received scant attention as yet. Data on the absorption of a few vegetable oils, normal, thermally treated, hydrogenated cocoanut oil and also of groundnut oil which has been subjected to different degrees of hydrogenation are presented in this communication.

EXPERIMENTAL

The absorption of oils by normal adult rats has been evaluated by balance method. The animals were at first maintained on the following fat free diet, 20 g. of which were given daily per a pair of rat, until a constant excretion of fat was obtained in the faeces. 2 cc. of the normal, thermally treated and hydrogenated oils were then mixed with 16 g of the diet, thereby keeping the calorific value

*Lady Tuta Memorial Scholar, 1943-44.

of the daily diet approximately constant. The feeding period was again continued until five consecutive readings, taken at intervals of three days, of the fat content of the faeces was found to be the same.

Composition of diet:

Sucrose	36%
Starch	36%
Casein	10%
Egg white	16%
Salt Mixture (Osborn and Mendell)	2%

Vitamin tablets (Complevite) containing vitamins A, B, C and D, were powdered and adequate amounts were added to this diet.

The oils were successively brought to 200°C, 250°C, 275°C and 300°C and maintained at those temperatures for one hour at the normal atmospheric pressure, with the only exception that at 300°C the oils were heated for a period of forty-five minutes only. The absorption of the following oils has been studied: groundnut oil, coconut oil, mustard oil, sesame oil, linseed oil and cow and buffalo ghee. All the oils were commercial products. The samples of cow and buffalo ghees were found, on analysis, to be adulterated.

The chemical properties and the percentage of absorption of the normal, thermally treated and hydrogenated oils are collected in Table I.

TABLE I.

Oil.	Temp. °C	Saponification value.	Iodine value.	Acid value.	Percentage of absorption.
Groundnut	Normal	184	83.5	9.6	99.7
	200	184	81.5	7.2	99.7
	250	186.5	83.0	5.3	99.7
	275	190	84.2	2.8	99.7
	300	192.5	76.0	2.5	99.5
Coconut	Normal	250	10.0	3.6	98.4
	200	246	10.0	3.4	98.3
	250	246	10.0	1.1	98.3
	275	246	7.4	0.5E	96.4
	300	240	6.6	...	91.9
Mustard	Normal	174	100.0	5.4	95.5
	200	172	99.5	3.8	95.2
	250	172	92.6	3.3	95.2
	275	170	87.0	1.8	91.7
	300	166.5	78.0	...	88.7
Sesame	Normal	189	109	13.4	96
	200	189	107	11.2	96
	250	187	96	4.4	95
	275	169	88	1.3	94
	300	190	83	...	92.5

Oil.	Temp. °C.	Saponifica- tion value.	Iodine value.	Acid value.	of absorption.	
Linseed	Normal	192	187	2.6	97	
	200	191	174	2.4	95.9	
	250	189	144	2.2	94.0	
	275	187	136	...	90.8	
	300	187	98	...	78.8	
Cow Ghee	Normal	197	56.5	1.8	99.2	
	200	199	56.7	0.8	98.2	
	250	196	50.8	0.5	97.0	
	275	199	48.0	...	96.4	
	300	199	42.7	...	90.6	
Buffalo Ghee	Normal	195	61.0	2.6	98.8	
	200	197.5	60.8	2.4	98.6	
	250	195	56.7	1.8	97.8	
	275	199	50.0	1.8	93.5	
	300	197	46.8	...	85.8	
Hydrogenated groundnut	Normal	191	69.5	...	99.5	
				(Soft)	...	99.5
				(Medium)	...	99.5
				(Hard)	...	99.5
Hydrogenated cocoanut	Normal	255	7.2	...	98.5	

It will be seen from the Table I that thermal treatment changes the character of most of the oils, both chemical and physiological, very significantly. The saponification values of the different thermally treated oils deviate but very little from normal. The acid and iodine values are, however, greatly affected. Iodine values of groundnut and cocoanut oils are not altered at 200°C or 250°C, but at 275°C the iodine value of cocoanut oil drops down to 7.4, while the iodine value of groundnut oil is not altered even at this temperature. At 300°C, however, the iodine value of this oil drops to 76, the initial value being, 83.5. Iodine value of linseed oil begins to change at 200°C—the initial value is 187 and at 200°C, it becomes 174—begins to fall more markedly at successive increases of temperatures, until at 300°C, the iodine value stands at 98. The iodine value of sesame and mustard oils and the adulterated samples of ghees begin to fall at 250°C and with gradual rise of temperature, the decreases in iodine values become more marked.

The physical changes noticed were a change of colour and viscosity. With the exception of the two ghees, the oils assume a reddish brown tinge. There is a gradual rise in the viscosity of the oils with the increase of temperature; linseed oil when treated to 300°C becomes almost solid at room temperature. The viscosity of ground-nut oil changes very slightly when compared with the other oils.

Thermal treatment up to a temperature 250°C does not affect absorption of the different oils, with the exception of linseed oil. But treatment at 275°C and 300°C lowers the absorption of all the oils, excepting groundnut oil. The percentage of decrease in absorption due to thermal treatment is, however, not uniform. The absorption of cocoanut and sesame oil is slightly lowered, that of mustard oil and adulterated ghees is more marked. The reduction in absorption of linseed oil due to thermal treatment is, however, the most marked. The absorption of this oil, unlike all others, begins to fall when this oil is treated to 250°C. Groundnut oil, however, behaves very uniquely; its absorption is not at all affected by thermal treatment.

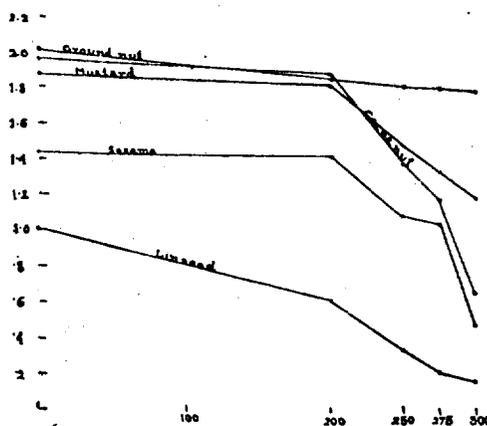


Fig. 1.

Abscissa=temperature in centigrades.

Ordinate=cc. of N/10 free acid liberated in one hour per every cc. of the incubating mixture.

Hydrogenation of groundnut and cocoanut oils to different degrees does not either affect the absorption or the saponification values of these oils—it is characterised by a gradual fall of iodine value. It is evident, therefore, that the degree of unsaturation does not affect the absorption of oils. This observation also confirms the finding of Peretti and Reale (2).

The study of the rate of hydrolysis of normal and thermally treated oils by lipase *in vitro*, is however quite interesting (Table II).

A mixture of the following composition was incubated at room temperature. 1 cc. of it was withdrawn at zero and one hour and delivered into Erlenmayer flasks containing alcohol-ether mixture. The flasks were then kept for five minutes in a boiling water-bath, cooled and titrated with 0.05 N-alcoholic potash to tritrate the free acidity of the mixture, (fig I).

TABLE II.

Composition of the incubating mixture:		
0.1 g. Lipase (Frenkel and Landau)	...	in 1 cc. water.
0.05 g. Na-Taurocholate (Difco)	...	in 1 cc. "
5% Albumin	0.5 cc.
2% CaCl ₂	0.5 cc.
Glycine-NaOH buffer (pH 8.8)	...	1.0 cc.
Oil	1.0 cc.
Total volume	...	5.0 cc.

Oil.	Temp. °C.	Amount of fatty acid liberated in one hour per every cubic centimetre.
Groundnut	Normal	2.04 cc. N/10 acid
	200	1.87 "
	250	1.88 "
	275	1.83 "
	300	1.81 "
Coconut	Normal	1.96 "
	200	1.89 "
	250	1.40 "
	275	1.20 "
	300	0.68 "
Mustard	Normal	1.87 "
	200	1.88 "
	250	1.50 "
	275	1.36 "
	300	1.21 "
Sesame	Normal	1.44 "
	200	1.44 "
	250	1.11 "
	275	1.06 "
	300	0.51 "
Linseed	Normal	1.01 "
	200	0.68 "
	250	0.36 "
	275	0.24 "
	300	0.18 "

It may be noted from Table II that with the increase of thermal treatment, there is a progressive decline in the rate of hydrolysis of the oils. It was observed that the more viscous oils could not be emulsified to the extent it was possible to emulsify the less viscid oil. It is highly probable that the lowered rate of hydrolysis of the more viscous oil is due to the great difficulty experienced in emulsifying these samples and that the degree of emulsification depends on the viscosity of the oils; it is also evident from Tables I and II that there exists a close parallelism between the lowered rate of hydrolysis and decreased absorption of the oils. The rate of hydrolysis of the normal oils is also not uniform and the

present author is of opinion that this difference may be due to the difference in the number of easily hydrolysable linkages present in the oils.

In summing up, it may be remarked, therefore, that the oils which become more viscid when subjected to thermal treatment are emulsified to a lesser extent and this in its turn lowers the rate of hydrolysis and, therefore, their absorption.

SUMMARY

1. The percentage of absorption of a few normal, hydrogenated and thermally treated oils has been evaluated by balance method.
2. A few changes in the chemical and physical properties of the oils brought about by thermal treatment have been studied.
3. The degree of unsaturation does not affect absorption.
4. Excepting groundnut oil the absorption of all other oils decreases when they are subjected to thermal treatment.
5. Evidences have been presented to show that the increase in the viscosity of the oils due to thermal treatment reduces the activity of emulsifying agents, thereby retarding lipase activity and absorption.

I should like to express my thanks to Dr. B. B. Sircar and Mr. P. B. Sen for their kind interest and to the Lady Tata Memorial Trust for the award of a scholarship. Thanks are also due to Tata Oil Mills, Cochin and H. V. M. Co., Bombay for a gift of crude and hydrogenated groundnut and coconut oils.

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Six adult, litter-mate rats were fed on a diet similar to that used by Basu and Nath excepting for the use of casein in the place of fish. The diet analysis showed 9.98 per cent. protein, 7.44 per cent. fat, 0.093 per cent. calcium (Ca) and 0.158 per cent. phosphorus (P). Collection of urine, faeces and diet was made for a period of four days and for comparison, *ghee* (clarified butter) was used in the place of coconut oil. The results of the experiment are presented in Table I.

TABLE I
Nitrogen, calcium and phosphorus balance in rats
fed on coconut oil and ghee

Fat	In diet mg.	In urine mg.	In faeces mg.	Balance mg.
Nitrogen				
Coconut oil	790.0	279.0	177.7	333.3 ± 39.99
Ghee	834.7	258.9	202.9	372.9 ± 18.30
Phosphorus				
Coconut oil	78.2	29.2	19.0	30.0 ± 5.28
Ghee	82.6	29.0	20.6	33.0 ± 4.05
Calcium				
Coconut oil	46.10	2.22	15.02	28.86 ± 4.35
Ghee	48.70	2.16	12.52	34.02 ± 2.11

A positive calcium balance was noticed in all cases and there was no appreciable difference between coconut oil and *ghee* as far as the assimilation of nitrogen, calcium and phosphorus are concerned.

Grateful thanks are due to the Parlakimedi Trust of the Indian Research Fund Association for the award of a scholarship.

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October 16, 1949.

THE INFLUENCE OF THE INTAKE OF COCONUT OIL ON CALCIUM BALANCE

The relationship between calcium utilization and intake of coconut oil is important because it is almost the only oil consumed by certain classes in South India. If, as the results of Basu and Nath¹⁻³ would show, intake of coconut oil is associated with a negative calcium balance, diseases due to calcium deficiency should have been widely prevalent. Since this has not been reported so far, it was felt necessary to re-examine this problem.

¹ Basu, K. P., and Nath, H. P., *Indian Journal Med. Research*, 1940, 34, 13. ² —, *Ibid.*, 1946, 34, 19. ³ —, *Ibid.*, 34, 27.

EFFECT OF OLEIC ACID ON THE DELAY OF SEXUAL MATURITY IN
CHICKENS CAUSED BY *STERCULIA FOETIDA* OIL¹

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It has recently been shown that feeding 200 mg. of *S. foetida* oil per day to sexually maturing pullets resulted in retardation of comb, ovary, and oviduct development

¹Arizona Agricultural Experiment Station Technical Paper No. 711.

(Schneider *et al.*, 1962). Also noted in these experiments were lack of egg production, enlargement of the gall bladder and liver, and a decrease in the iodine number of the omental fat. Evans *et al.* (1961) showed that feeding either cottonseed oil

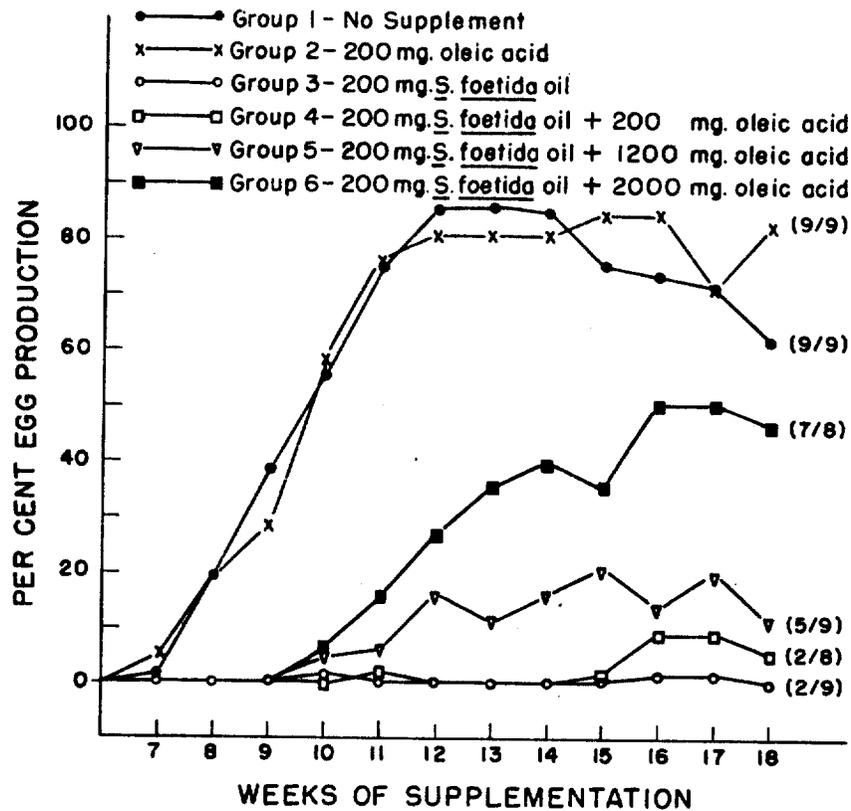


FIG. 1. Percent egg production during 18 weeks of supplementation (Numbers at the end of each line indicates the number of pullets laying and number in the group).

or *S. foetida* seeds (both of which contain Halphen-reactive cyclopropene fatty acids) to laying hens produced eggs which had a higher percentage of stearic acid and a lower percentage of oleic acid.

Both of these investigations indicated that the cyclopropene fatty acids apparently interfered in some manner with the conversion of stearic acid to oleic acid even though they are present only in small amounts. What role this metabolic derangement might have on sexual maturity is unknown. However, Panos *et al.* (1959) and others have shown a relationship between the pituitary-gonadal complex and lipid metabolism.

The following experiment was designed to determine if added oleic acid would

overcome the detrimental effect of *S. foetida* oil on pullets using egg production as a criterion for ovary and oviduct development.

Fifteen weeks old S. C. White Leghorn² pullets were randomly distributed into 6 groups of 9 birds each and maintained in individual laying cages. Water and a practical type diet were supplied *ad libitum*. The diet was the same as that reported by Schneider *et al.* (1961) except that 1% added hydrolyzed animal-vegetable fat was replaced with glucose monohydrate.³ The diet contained approximately 2.2% total fat. The hens in each group were individu-

² Kimber strain 137.

³ Cerelose, Corn Products Co., New York.

ally supplemented daily by gelatin capsule as follows: group 1, empty capsule; group 2, 200 mg. of oleic acid;⁴ group 3, 200 mg. of *S. foetida* oil; group 4, 200 mg. of *S. foetida* oil plus 200 mg. of oleic acid; group 5, 200 mg. of *S. foetida* oil plus 1200 mg. of oleic acid; and group 6, 200 mg. of *S. foetida* oil plus 2000 mg. of oleic acid.

Figure 1 shows the percent egg production of the groups during 18 weeks of supplementation. Both control groups, 1 and 2, started to lay by the seventh week of supplementation (age 22 weeks) and were in full production by the twelfth week (age 27 weeks), considered normal for this strain of pullets. None of the *S. foetida* oil treated groups started to lay until the tenth week of supplementation (age 25 weeks). When *S. foetida* oil was fed alone (group 3), only 2 of the 9 birds layed and then only an occasional egg throughout 18 weeks of supplementation. As the level of oleic acid was increased (groups

4, 5 and 6) better production was noted and group 6 (200 mg. *S. foetida* oil-2000 mg. oleic acid daily) reached 50% production by the sixteenth week (age 31 weeks).

Thus, oleic acid supplementation partially alleviated the delay in sexual maturity and egg production caused by *S. foetida* oil. Whether a higher level of oleic acid would allow better egg production must still be determined. Also unanswered is the possible mode of action of oleic acid on the pituitary-ovary complex, since onset of egg production was delayed 3 or 4 weeks. There is also the possibility that one of the contaminants in the oleic acid used may act as the alleviating agent.

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⁴ Nutritional Biochemical Corp., Cleveland. Tentative analysis by gas chromatography shows it to contain approximately 71-75% oleic acid. Other fatty acids possibly present are myristic, myristoleic, palmitic, palmitoleic, stearic, linoleic and linolenic.

THE EFFECT OF OLIVE OIL AND OLEIC ACID ON GASTRIC SECRETION IN THE RAT. By R. SCHNEIDER. From the Department of Medical Biochemistry and Pharmacology, The Medical School, Edgbaston, Birmingham, 15.

(Received for publication 21st May 1958)

In the rat the inhibitory effect of olive oil on spontaneous gastric secretion is weak and erratic. Large amounts of oil have to be used. Previous hydrolysis of the oil markedly enhances the inhibitory effect. Oleic acid and sodium oleate are potent inhibitors of both spontaneous and insulin-stimulated gastric secretion.

Draining the upper intestinal juices reduces, but does not abolish, the inhibitory effect of sodium oleate on insulin-stimulated gastric secretion. Distension of the duodenum with liquid paraffin reduces spontaneous as well as insulin-stimulated gastric secretion. Distension of the jejunum or ileum does not have this effect. Distension of the duodenum with sodium oleate as well as with water reduced gastric secretion. There is a positive correlation between the inhibitory effect and the distending pressure. At the same pressure sodium oleate is more potent in depressing gastric secretion than is water.

THE inhibitory effect of fat on gastric secretion has been known since the classical experiments of Beaumont [1838] and Ewald and Boas [1886]. Further experimental work by Szokolov [1904] and Lönnquist [1906] showed that the effect was due to contact of fat with the mucosa of the small intestine. Products of lipolysis such as fatty acids and soaps were supposed to have a stimulating effect on gastric secretion. This view was based on the experiments of Piontkowsky [1904] and Babkin [1905], who showed that the introduction of sodium oleate through the duodenal fistula of a dog caused secretion from "the little stomach", and on the experiments of Ivy and McIlvain [1923-4], who obtained secretion from dogs with fundic pouches after the introduction of short chain fatty acids and "Ivory Soap" into a Thiry-Vella loop.

This generally accepted view was first challenged by Roberts [1931] and Shay, Gershon-Cohen and Fels [1939]. Roberts showed in experiments on man that not only neutral fat, but also free fatty acids, inhibited gastric secretion; Shay, Gershon-Cohen and Fels—also in man—produced marked depression of secretion during and after the intraduodenal instillation of a solution of sodium oleate. Recently Sircus [1958] working with dogs obtained a depression of gastric secretion with the intraduodenal instillation of olive oil incubated with pancreatic juice.

Experiments on dogs [Quigley and Meschan, 1941] and man [Card, 1941] showed that soaps and free fatty acids also depressed gastric motility, and that the products of lipolysis were often more potent inhibitors than the corresponding neutral fat.

Clinical data also point to the greater potency of the products of lipolysis in depressing gastric function. Both in tropical sprue and idiopathic steatorrhœa depression of gastric secretion is a frequent if erratic symptom [v.d. Scheer, 1905; Baumgartner and Smith, 1927; Serra, 1929; Fairley, 1930; Hess Thaysen, 1932; Frazer, 1950] and according to Frazer [1949] decreased gastric motility also is a marked feature of the sprue syndrome. In this syndrome particulate absorption of triglycerides is at fault, and abnormal quantities of fatty acids may occur in the intestine [Frazer, 1950].

The present investigation was undertaken in order to determine the relative potency of neutral fat and certain products of lipolysis in inhibiting gastric secretion in the rat.

METHODS

Operative Procedures.—White male rats weighing 200–300 g. were used. The night before the experiment all solid food was removed and the rats given a 5 per cent glucose solution to drink. In some of the experimental series 2½ per cent casein hydrolysate was added to the glucose solution.

On the day of the experiment a laparotomy was performed under ether anaesthesia, the stomach exposed and a small incision made just proximal to the pylorus. Great care was taken not to injure the blood vessels during this procedure. Two glass cannulae of 2 mm. internal and 3 mm. external diameter, with a rectangular bend, were introduced through this incision and firmly secured by ligatures. One cannula was introduced caudally through the pylorus into the duodenum, the other pointing orally into the stomach. Both cannulae carried a length of rubber "drainage" tubing of 1 mm. internal diameter. The tubing on the duodenal cannula was about 45 cm. in length. The distal end was tied, and the whole tubing and cannula filled with the substance under investigation. The tube on the gastric cannula was about 1 cm. long and left open. The purpose of the gastric cannula was to allow the stomach to be emptied at the beginning of the experiment and any food residue to be washed out. The abdominal incision was closed with a continuous suture and the wound painted with celloidin. The tubing of the gastric cannula was then closed with a small artery clip (fig. 1). The rats were put into single wire cages with wire bottoms to prevent coprophagy. The closed end of the duodenal tube was taken through the top of the cage and secured with an artery clip. The rats recovered from the anaesthetic after a few minutes and were able to move about the cage. All injections were given into the duodenal tube without disturbing the rats. At the end of the experiment the rats were sacrificed, the stomach contents aspirated, the volume measured, the pH determined with indicator papers, the free acid titrated with N/100 NaOH, using methyl orange as indicator, and the total chlorides estimated according to Patterson's micro-technique [Harrison, 1943]. In some of the experiments the volume of the small intestine from pylorus to ileocaecal valve was measured and the pH again determined with indicator papers.

In a few of the experiments rats were fitted with a duodenal fistula in order to drain the upper intestinal secretions. A glass cannula of the same design as that used for the stomach was introduced through a small incision in the anti-mesenteric border of the duodenum well distal to the entry of the bile duct and firmly tied in. A glass reservoir of about 3 ml. capacity was attached to the cannula with rubber tubing so

that the drained fluid could be collected. A second cannula was introduced immediately distal to the first, through which the substances under investigation could be administered in the usual way.

Method for obtaining a Controlled Fluid Pressure.—A ligature was tied round the duodeno-jejunal flexure. A duodenal cannula was introduced through the pylorus and connected with a glass reservoir of 7 mm. diameter. The whole system was completely filled with the material under investigation, and the top of the reservoir set at the required height above the bottom of the cage. Any increase of intraduodenal pressure was compensated by the spilling of fluid over the top of the reservoir and any reduction of pressure by refilling the reservoir to the original level.

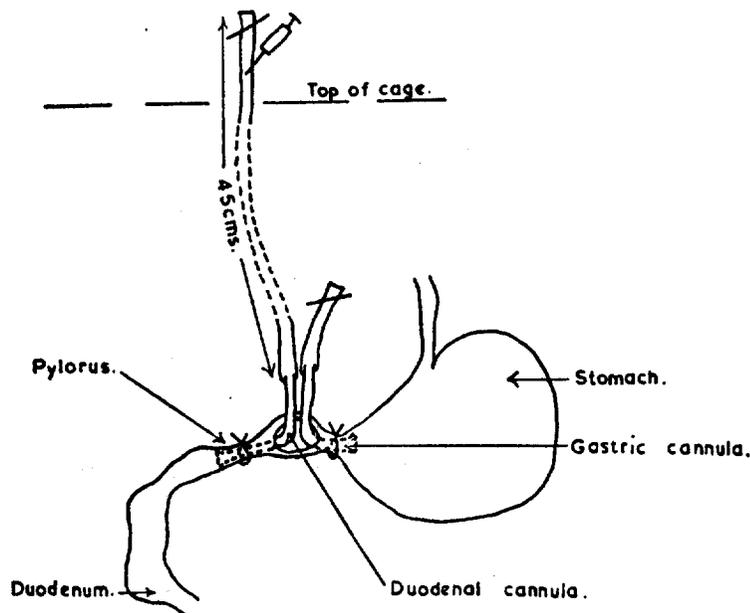


FIG. 1.—Diagram of the operative procedure.

Hydrolysis of Olive Oil.—Olive oil buffered at a pH of 7.8 was shaken at 37° C. with bile salts and pancreatin for 12–24 hr., and the resultant hydrolysate extracted with ether. The ether was evaporated and the residue used.

An analysis of the hydrolyzed olive oil showed the following composition: triglyceride 18 per cent, diglyceride 10 per cent, monoglyceride 10 per cent, free acid 62 per cent.

RESULTS

The Effect of Olive Oil on Spontaneous Gastric Secretion.—Unemulsified undiluted olive oil (BP) was introduced intraduodenally in amounts of 45 mg. every 20 min. over a period of 6 hr. The control animals received the corresponding amount of water. The results are shown in Table I. Under these conditions olive oil did not have any inhibitory effect on gastric secretion. On the contrary, the mean values for both free acid and total chlorides were raised. The difference between the means of controls and tests, however, was not significant at the 0.05 level. The amount of olive oil was therefore increased. Only when 180 mg. were used a significant depression of secretion resulted.

After previous hydrolysis olive oil became a potent inhibitor of gastric secretion. Amounts as small as 26–44 mg. caused a significant depression of free acid as well as total chloride secretion.

TABLE I.—EFFECT OF UNHYDROLYZED AND PREHYDROLYZED OLIVE OIL ON SPONTANEOUS GASTRIC SECRETION

Duration of experiment: 6 hr.

Substance	Amount in mg./20 min.	No. of rats	Mean total chloride in m.equiv.	Mean total free HCl in m.equiv.	S.E.	t	P
Controls (water 0.1–0.2 ml.)		14	0.84		±0.083
		18		0.51	±0.066
Olive oil	45	6	1.070		±0.280	1.045	0.4–0.3
		6		0.690	±0.210	1.120	0.3–0.2
	180	8	0.275		±0.099	4.280	< 0.001
Prehydrolyzed olive oil.	26–44	8		0.078	±0.046	4.100	< 0.001
		5	0.260		±0.071	3.980	< 0.001
		5		0.070	±0.071	3.300	< 0.001

The Effect of Oleic Acid and Sodium Oleate on Spontaneous Gastric Secretion.—As olive oil after hydrolysis had an inhibitory effect, oleic acid, its main constituent fatty acid, was administered under the same conditions. Oleic acid was used both in the free form and as the sodium soap in a 10 per cent solution. The results are shown in Table II. With oleic acid depression

TABLE II.—EFFECT OF OLEIC ACID AND SODIUM OLEATE ON SPONTANEOUS SECRETION

Duration of experiment: 6 hr.

Substance	Amount in mg./20 min.	No. of rats	Mean total chloride in m.equiv.	Mean total free HCl in m.equiv.	S.E.	t	P
Controls (water 0.1–0.2 ml.)		14	0.840		±0.083
		18		0.510	±0.066
Oleic acid	10–20	6	0.470		±0.094	2.450	0.05–0.02
		6		0.280	±0.049	1.930	0.10–0.05
	45	9	0.040		±0.011	7.760	< 0.001
Sodium oleate	5–10	9		0.000	±0.000	5.370	< 0.001
		7	0.31		±0.087	4.000	< 0.001
	15–30	8		0.100	±0.043	3.920	< 0.001
		5	0.00		±0.000	3.250	< 0.01
	5		0.000	±0.000	4.000	< 0.001	

of both free acid and chloride was obtained with amounts as small as 10–20 mg., and an increase of the dose to 45 mg. completely suppressed acid secretion. The sodium soap in even smaller amounts (5–10 mg.) depressed secretion and in amounts of 15 mg. or over caused complete suppression.

The Effect of Oleic Acid and Sodium Oleate on the Secretory Response to Insulin-induced Hypoglycemia.—The results are shown in Table III. The amount of insulin used was 0.15 unit/100 g. body weight. With this amount toxic symptoms were avoided and the secretory response of the stomach after 3 hr. was of the same order as the spontaneous secretion after 6 hrs. Both

sodium oleate 20 mg. and oleic acid 45 mg., given every 20 min., again produced a significant depression of both chloride and free acid secretion.

TABLE III.—THE EFFECT OF OLEIC ACID AND SODIUM OLEATE ON GASTRIC SECRETION STIMULATED BY INSULIN INDUCED HYPOGLYCAEMIA

Duration of experiment: 3 hr.

Substance	Amount in mg./20 min.	No. of rats	Mean total chloride in m.equiv.	Mean total free HCl in m.equiv.	S.E.	t	P
Controls (water 0.1-0.2 ml.)		22	0.75		±0.09
		22		0.53	±0.08
Oleic acid	45	4	0.20		±0.008	2.44	0.05-0.02
		5		0.10	±0.054	2.57	0.02-0.01
Sodium oleate	20	12	0.075		±0.026	6.34	< 0.001
		13		0.023	±0.012	4.97	< 0.001

The Effect of Draining the Upper Intestinal Juices on the Inhibition of Gastric Secretion by Sodium Oleate.—The same experimental procedures were carried out on rats that had been fitted with duodenal fistulae in order to study the importance of the upper intestinal juices on the inhibitory mechanism. This time sodium oleate was used and only free acid determined. The results are shown in Table IV. Although the intraduodenal administration of sodium oleate still caused inhibition of gastric secretion, the degree of depression compared with that in the animals without duodenal fistulae had been significantly reduced ($t: 3.1$; $P: 0.01-0.001$).

TABLE IV.—THE EFFECT OF SODIUM OLEATE ON GASTRIC SECRETION STIMULATED BY INSULIN INDUCED HYPOGLYCAEMIA

Duration of experiment: 3 hr.

Substance	Amount in mg./20 min.	No. of rats	Type of preparation	Mean total free HCl in m.equiv.	S.E.	t	P
Controls (water)	20-50	14	Duodenal fistula	0.596	±0.089
Sodium oleate	20	19	Duodenal fistula	0.302	±0.076	2.5	0.02-0.01

The Effect on Gastric Secretion of Distending the Small Intestine with Liquid Paraffin.—In order to assess what effect distension of the small intestine alone might have on gastric secretion, liquid paraffin was introduced intraduodenally in a single amount of 5 ml. and a ligature tied immediately beyond the material administered. The effect was studied on spontaneous secretion (fig. 2) as well as on insulin stimulated secretion (Table V). Spontaneous secretion was completely suppressed, and the response to insulin induced hypoglycaemia significantly reduced.

When the site of administration was changed to the middle of the jejunum, or to the more distal portion of the ileum, no inhibition of gastric secretion resulted (Table V).

Comparison of the Inhibitory Effect of Olive Oil on Gastric Secretion Stimulated by Insulin-induced Hypoglycaemia with that of Liquid Paraffin.—In this series of experiments olive oil and liquid paraffin were administered intra-

duodenally in equal amounts. The rats were fitted with duodenal fistulae to avoid the effect of the additional volume of upper intestinal juices. The amount of oil was 270 mg. every 20 min. for 3 hr. and insulin-induced hypoglycaemia was again used as the stimulus for gastric secretion. The results

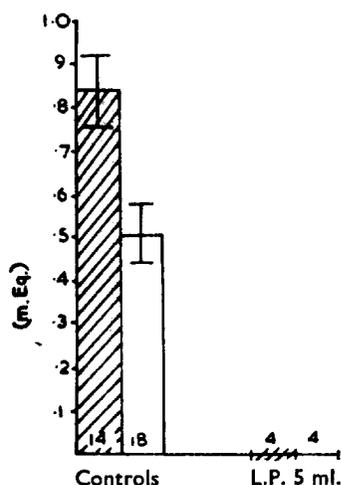


FIG. 2.—The effect of the intra-duodenal administration of a single dose of liquid paraffin on spontaneous gastric secretion in conscious rats. Pylorus and duodenum ligated. Duration of experiment 6 hr. The hatched columns represent the means of total chlorides and the clear columns the means of the free acid secreted. The bars represent the standard errors of the means. The numbers refer to the numbers of animals used. L.P. = liquid paraffin.

with olive oil were erratic. Complete or almost complete depression of free acid secretion occurred with three of the six rats examined. With liquid paraffin depression of secretion occurred in four of the six rats used and there was no significant difference between the means ($t: 0.32$). Doubling the amount of liquid paraffin did not enhance its inhibitory effect.

TABLE V.—THE EFFECT OF DISTENSION WITH A SINGLE DOSE OF 5 ML. OF LIQUID PARAFFIN ON GASTRIC SECRETION STIMULATED BY INSULIN INDUCED HYPOLYCAEMIA.

Site of distension	No. of rats	Duration of experiment: 3 hr.		S.E.	t	P
		Mean total chloride in m.equiv.	Mean total free HCl in m.equiv.			
Controls (no distension)	22	0.75		±0.09
	22		0.53	±0.08
Duodenum	7	0.12		±0.05	4.56	< 0.001
	7		0.06	±0.04	3.33	< 0.001
Jejunum or ileum	7	0.55		±0.09	1.14	0.3-0.2
	7		0.44	±0.10	0.73	0.5-0.3

The Effect of Pressure Changes in the Duodenum on the Inhibitory Effect of Distension on Gastric Secretion. (Comparison of the Effect of Sodium Oleate with that of Water.) In these experiments a ligature was tied around the duodeno-jejunal flexure, the duodenum was distended with sodium oleate and the distending pressure kept constant throughout the experimental period of 3 hr. Insulin-induced hypoglycaemia was used as the stimulus for gastric secretion. Fig. 3a shows a significant correlation between the distending pressure and the inhibition of gastric secretion ($r: -0.622$; $P: 0.01-0.001$). When water was used instead of sodium oleate (fig. 3b) a

significant correlation ($r: -0.516$; $P: 0.05-0.02$) was again found, but water was less potent in depressing gastric secretion than sodium oleate.

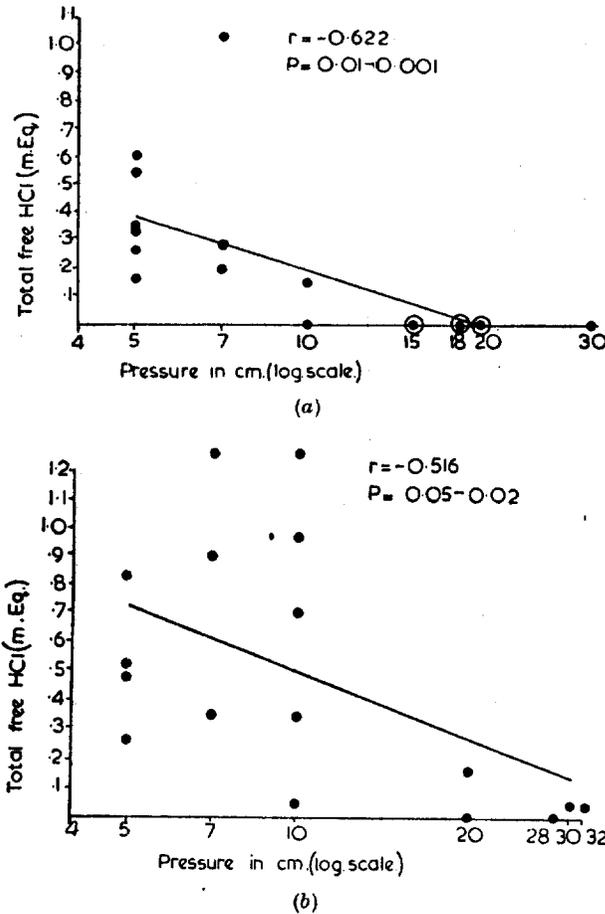


FIG. 3.—The effect of intraduodenal pressure on gastric secretion of free HCl by conscious rats. The pressure is expressed as the height of the fluid level above the wire bottom of the cage (log. scale). r : correlation coefficient. The lines are calculated regression lines.

(a) the effect of 10 per cent sodium oleate.

(b) the effect of water.

DISCUSSION

Piontkowsky [1904] and Babkin [1905], and recently Sircus [1958] administered fatty acid intraduodenally into dogs and found a stimulation of gastric secretion. The reason for the difference in the two species is not clear. Unfortunately, neither the concentrations of the solutions nor of the exact position of the fistulae are stated by the earlier workers. A possible stimulating effect of weaker concentrations would be more easily detected in dogs,

who frequently have no fasting gastric secretion [Babkin, 1950; Neeheles, 1955] than in rats, who secrete large amounts of acid gastric juice when fasting.

Since the experiments of the Pavlov school [Szokolov, 1904 and Lönnquist, 1906] it has been accepted that the inhibitory effect of fat on gastric secretion is due to its presence in the intestine. During the present investigation it was found that inhibition of gastric secretion was frequently associated with the accumulation of large amounts of fluid in the intestine at the end of the experimental period. This suggests that the upper intestinal juices might play a part in the inhibitory mechanism. Draining the upper intestinal juices significantly reduced, but did not abolish, the inhibitory effect of sodium oleate. Under these conditions the volume of the intestinal fluid found at the end of the experiment was reduced, but was still larger than that of the control animals. These findings suggest that distension of the intestine with fluid might be an important factor in the inhibitory mechanism of fat on gastric secretion.

The inhibitory effect of distension of the upper small intestine on gastric secretion in dogs was first mentioned by Day and Webster [1935] and later shown to vary with the degree of the distending pressure [Sircus, 1953; Walker, 1955]. It is well known that hypertonic solutions of glucose are potent inhibitors of gastric secretion in man and dog [Muir, 1949], and it seems likely that the superiority of intraduodenal hypertonic glucose over that of intravenous glucose as an inhibitor of gastric secretion [Muir, 1949] is due to the duodenal distension it evokes.

In an attempt to demonstrate the effect of distension on gastric secretion, unemulsified liquid paraffin was introduced intraduodenally in the present investigation and prevented from passing down the intestine by a ligature just beyond the material administered. A significant inhibition of both spontaneous secretion and insulin stimulated secretion was found. With changing the site of administration to the jejunum or the ileum, no depression of gastric secretion occurred. These findings suggested that the inhibitory mechanism operated from the duodenum only and not from the more distal portions of the small intestine. If the distending material was allowed to pass down the intestine, depression of gastric secretion still occurred, but the results became erratic. There was no difference between the inhibiting effect of olive oil and liquid paraffin under these conditions. The fact that increasing the volume of the single dose of distending material from 0.3 ml. to 0.6 ml. did not increase the effect indicated that the distending pressure in the duodenum was a more important factor in this mechanism than the volume of fluid administered. This was confirmed by the finding of a negative correlation between distending pressure in the duodenum and the degree of free acid secretion by the stomach. Such a negative correlation was found with water, as well as with sodium oleate. Sodium oleate, however, seemed to inhibit gastric secretion more than water at the same distending pressure.

In the sprue syndrome several factors contribute to cause distension of the small intestine. Absorption is generally depressed and increased amounts of free fatty acids may be present in the intestine. Further small intestinal

motility is reduced and there is excess mucus secretion; also an increase of volatile fatty acids, due to fermentation, is a frequent occurrence. The radiological picture of the distended small intestine after the administration of non-flocculating barium is one of the characteristic features of the syndrome.

The present findings in the rat suggest that such distension of the duodenum might be a major factor in producing hypochlorhydria or achlorhydria as they frequently occur in the sprue syndrome, and might also account for the erratic nature and the reversibility of these symptoms.

ACKNOWLEDGMENTS

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SOME OBSERVATIONS UPON THE BEHAVIOR OF A
FIXED OIL (PEANUT OIL) INJECTED
INTRAPERITONEALLY

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This study was made incidentally to the investigation of another problem in which fixed oil was injected into the peritoneal cavity as a solvent for fat soluble material. Although it has long been known that when Linamentum Camphorae, U. S. P., a preparation of cottonseed oil and camphor intended for external use only, is injected into the subcutaneous tissue it may remain there for a long time and is prone to lead to pathological processes (1), very few data concerning the behavior of fixed oil within the peritoneal cavity exist. Corper (2) injected into the peritoneal cavity of guinea-pigs solutions of Sudan III in oil or butter, and upon performing post mortem examination observed the following: The presence of much of the injected oil free within the peritoneal cavity; a superficial staining of the abdominal fat, with the exception of that at the head of the testes: fibrin-like masses around the liver and under the diaphragm; and stained oil in some of the lymph glands.

In the experiments herein reported, from 1 to 10 cc. of cold pressed peanut oil was injected into the peritoneal cavity of rats mice, guinea-pigs, rabbits and cats, under clean but not aseptic technique. A few animals received oil containing 1 per cent lecithin, and a few more received oil containing Sudan III (C. P. melting point 200°C. uncorrected).¹ The animals were well fed and at no time was the effect of inanition upon the absorption.

¹ This material was prepared in the Color Laboratory of the Bureau of Chemistry.

of the oil tested. After their general behavior was noted for varying lengths of time the animals were killed and post mortem examination performed. The amount of oil remaining within the peritoneal cavity, the pathological effect of the oil upon the peritoneum, the intensity of staining of different parts of the peritoneum and the disappearance of dye both from the oil and from the stained parts were determined by inspection. In two instances the percentage of dye in the free oil remaining in the peritoneal cavity was determined in a Dubosque colorimeter.

As far as was observed no untoward physiological effect was produced in any of the animals by the oil. The rats grew well, pregnancy occurred and a few litters were raised. There was never any oil or dye noted in the region of the vagina. Rabbits and guinea-pigs remained in good health, a point which is particularly interesting in view of the reputed harmfulness for these animals of a diet containing unsaturated fatty acid esters. Mice and cats seemed to be unharmed by the oil.

RESULTS FROM POST MORTEM EXAMINATION OF ANIMALS

Rats. Two and three months after the injection, a considerable amount of the oil which had been injected usually was found free within the peritoneal cavity. The use of 1 per cent lecithin in the oil did not seem to favor materially its rate of disappearance from the peritoneal cavity. Each of eight rats, which received 8 cc. of oil containing 0.2 per cent Sudan III, were examined after a period of twenty-four weeks, in one no oil was found, in another only a film of oil covered the peritoneum, while in the remaining six varying amounts of free oil were present. In several of these rats chains of oil globules were found retroperitoneally, running in the general direction of the lumbar nerves.

The superficial character of the staining of the fatty deposits, in the rats which received the oil containing the dye, was very apparent in cross section of the thicker parts. Although the staining was relatively greater the more concentrated the dye, the most noticeable feature was the different degree or intensity of staining of various parts. The omentum, broad ligament and

mesentery were the most deeply colored. The margin of the mass of fat attached to the testicle was next in order, while the base or pedicle of the latter, together with the lumbar fat, was the least stained. The peritoneum over the abdominal muscles also appeared slightly stained. Although no observations were made as to the rate at which the stained oil or dye was first taken up, a fading of the intensity of the staining of the peritoneum was plainly noticeable at the beginning of the third month. The samples of free oil from two rats which were killed at the end of the twenty-fourth week were found to have suffered a loss in dye, as determined in the colorimeter, of 85 and 95 per cent, respectively.

The least noticeable feature to the inexperienced eye, although a very important one, was the occurrence of single or flattened conglomerations of cyst-like spheres containing oil. The individual spheres varied in size, the largest being several millimeters in diameter. They usually became very evident by the end of the first month and were found loosely attached to the peritoneum, chiefly on margins of the liver and the dome of the diaphragm. Occasionally a single cyst attached by a long thread-like ligament was seen. At first the walls of these cyst-like bodies were very thin and transparent, but later became thicker and opaque. Their number and distribution, which varied in different individuals, were brought out very clearly by the use of the stained oil. By the twenty-fourth week they contained much semi-solid matter, while a few were distinctly gritty, suggesting a calcification process. At this stage it was still possible to express from these bodies the stained oil which appeared quite deeper in color than that found free in the peritoneal cavity. Minor histological study of these cyst-like bodies was made and the presence of a connective tissue capsule, capillaries and granular contents ascertained. The nature of their lining, however, was not determined.

Mice. These animals were injected only with the unstained oil. After one month a considerable amount of the injected oil remained free in the peritoneal cavity. The cyst-like bodies referred to were also noted.

Cats. A few cats were injected with oil or oil containing Sudan III. In contrast to the behavior of the rats and mice, the cats showed a tendency for a little serous exudation into the peritoneal cavity, in which the oil sometimes formed small droplets. In only one cat were a few cyst-like bodies found. In another cat, the injected oil was traced by means of the dye, from the base of the uterus up the lumbar lymphatics. In one cat, the oil found at the end of the 6th month had a distinctly rancid odor.

Rabbits and guinea-pigs. In these animals light yellow fibrin-like shaggy and cheesy material formed within twenty-four to forty-eight hours. There was also a small amount of serous exudate. These phenomena did not disappear by the beginning of the second month, and oil droplets were still present within the cheesy material, as well as in the serous exudate.

DISCUSSION AND CONCLUSIONS

Although the purpose of this investigation was accomplished in the demonstration of the apparent harmlessness and the relatively slow absorption of fixed oil injected into the peritoneal cavity, a more detailed study of the problem would seem to offer a very interesting field of investigation. The different intensity of staining of different parts of the peritoneum would seem to be closely related to, or even evidence of the absorption of the stained oil by and through the lymphatic channels. The disappearance of dye from the oil illustrates the fact that even for a substance as relatively inert chemically as Sudan III, the behavior of dissolved substances cannot necessarily be traced by following the behavior of the solvent oil alone. It was suggested by Dr. C. L. Alsberg that fibrin-like masses observed in rabbits and guinea-pigs and the cyst-like bodies noted in rats and mice presented certain similarities to the calcium and magnesium soap phenomena of the so-called "fat necrosis." This possibility was strengthened by the subsequent finding of gritty material in the cyst-like bodies observed in rats.

The usefulness as a pharmacological method of the intraperitoneal injection of fixed oil as a solvent for fat soluble substances

would seem to be limited, if the entrance of the dissolved material into the circulation is dependent upon the relatively rapid absorption of the solvent oil. This, however, does not imply that its use for administering volatile, serum-soluble and even suspended material might not be advantageous. Until more is known concerning this method, its employment should be accompanied by control experiments in which post mortem examinations are made.

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**Mechanism of Experimental Tumorigenesis.
I. Epidermal Hyperplasia in Mouse Caused by
Locally Applied Tumor Initiator and Dipole-
Type Tumor Promotor^{1,2}**

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SUMMARY

Laboratory-synthesized "membrane active agents," Span 60 (sorbitan monostearate) and its ethylene oxide derivative, Tween 60 (polyoxyethylene sorbitan monostearate), and Tween 20 (polyoxyethylene sorbitan monolaurate), produced changes in skin on back of the mouse analogous to those caused by corresponding technical products. The degree of hyperplasia was correlated to the tumor-promoting property of the agent, or to its absence. The skin changes produced by a potent tumor promoter of Tween 60 type, which were analyzed histologically and histoquantitatively, differed from those caused by similarly applied carcinogens methylcholanthrene (MCA) and 9,10-dimethyl-1,2-benzanthracene (DMBA). A single application with MCA or DMBA immediately produced toxic alterations. Reparative hyperplasia set in only after the latter became quiescent. Continuous MCA or DMBA treatment caused cellular and nuclear atypia and severe disturbances in organization and differentiation,

which increased progressively. Treatment with Tween 60 did not produce retrogressive alterations, but intense cell multiplication and high-degree hyperplasia commenced immediately. Continuous Tween 60 treatment caused neither cellular nor nuclear atypia, nor disturbances in organization; the amount of cells in differentiation increased significantly. Continuous treatment maintained the hyperplasia unchanged. After initial application of carcinogen, Tween 60 treatment evoked changes typical of carcinogens. Tween 20 (a weak tumor promoter) and Span 60 (an ineffectual compound) did not produce these alterations. The nature of tumor promotion after application of a pure dipole-type promoter is benign. Tumor promotion is not a phase of the carcinogenic process itself as these processes are contradictory. Tumor promotion is comparable to intense continuous reparative ("simple") cell multiplication.—J. Nat. Cancer Inst. 23: 925-951, 1959.

SINCE 1945 we have used certain "membrane active agents" in studying the mechanism of tumorigenesis (1). Technical dipole-type tumor promoters produce a high degree of epidermal hyperplasia in the mouse (2-5).

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³ Department of Chemistry, Institute of Technology, Helsinki, Finland.

The degree of hyperplasia is always positively correlated to the concentration and tumor-promoting property of the compound used (2, 5). When treatment with these substances is continuous, the hyperplasia remains unchanged for as long as 60 weeks (4). The tumor incidence depends on the frequency of application: The oftener an aqueous compound (in aqueous state) is applied to the skin, the greater the number of tumors (5, 6). Light and electron microscopic observations (7-9) gave consistent evidence that tumor promotion in the mouse skin should be regarded as comparable to benign, reparative cellular proliferation. This is corroborated by the classical discovery of Deelman's (10), in which the proliferation that occurs in wound healing promotes the development of skin tumors [*cf.* Mottram (11)].

On the basis of our previous studies we feel convinced that tumor promotion, under the experimental conditions used, is not carcinogenic. Further supporting evidence is the observation made in this laboratory that a large number of pure fatty acids, which form the lipophilic portion of the membrane-active agents, are in themselves effective tumor promoters when applied often enough to the mouse skin (12).

It is the object of the present study to demonstrate by light microscopy and also histoquantitatively (1) that under controlled conditions, laboratory-synthesized dipole-type compounds produce related changes in the epidermis of the back of the mouse; (2) that these changes completely differ from those produced by locally applied carcinogenic hydrocarbons and are positively correlated, in degree, to the respective tumor-promoting property (11); and (3) that the changes are, in general, closely related to those caused by a "nonspecific tumor promoter," oleic acid.

MATERIALS AND METHODS

All general procedures were similar to those used in our previous studies.

The experimental animals were white male mice of the stock strain used in this laboratory since 1945 (1-9). Our mouse strain is fairly resistant to tumors, and so far no spontaneous tumors have been observed. The animals were 2 to 3 months old at the start of the experiments. They received food and water *ad libitum*. Animals treated with carcinogens were kept in separate laboratory rooms. No mice were lost during the experiments.

Carcinogens.—9,10-Dimethyl-1,2-benzanthracene (DMBA)⁴ and 20-methylcholanthrene (MCA)⁵ (obtained from L. Light & Co., Colnbrook, England) were dissolved in reagent grade acetone; dosages: experiment A, single dose of 60 γ of carcinogen ("application"); experiment B, 60 and 0.06 γ , respectively, applied continuously once daily, Sundays excluded. Application was made carefully with a glass pipette.

⁴ *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[a]anthracene.

⁵ *Chemical Abstracts'* nomenclature: 3-methylcholanthrene.

Secondary treatment.—The technical (t.) surfactants (Atlas Powder Co., Wilmington, Del.) were: Span 60 (sorbitan monostearate)—no tumor-promoting property (5, 13); Tween 60 (polyoxyethylene sorbitan monostearate)—an effective tumor promoter (2, 5, 6, 13-15); Span 20 (sorbitan monolaurate)—a rather weak tumor promoter (6); and Tween 20 (polyoxyethylene sorbitan monolaurate)—a poor tumor promoter (5, 13). Besides their various homologues, these technical detergents contain other fatty acids in addition to those stated by the manufacturer. The pure polyol fatty acid esters which were synthesized (s.) by us and which imitated the technical products were: Span 60 (sorbitan monostearate); Tween 60, derived from s. Span 60 by adding about 20 ethylene oxide units; Span 20 (sorbitan monolaurate); and Tween 20, derived from s. Span 20 [see (8)]. Of these compounds, s. Tween 60 [hydrophile-lipophile balance (HLB) value 14.9] and s. Tween 20 (HLB 16.7) are water soluble; s. Span 20 (HLB 8.6) is dispersible in water, and s. Span 60 (HLB 4.7) insoluble in water. Our intention had been to work with equimolar concentrations calculated from a 25 percent aqueous solution of s. Tween 60. However, owing to the nature of the compounds used, the molar concentrations are approximate only : 0.18 M. Treatment was given by painting the skin with a #16 hair brush, 6 and 12 times a week, continuously, Sundays excluded.

In experiment C the secondary treatment was given without the initial carcinogen application, and in experiment D secondary treatment was started 30 days after application of 60 γ MCA. In experiment E (without initial application of carcinogen) undiluted oleic acid (May & Baker Ltd., Dagenham, England) was applied 6 times a week, Sundays excluded. The treated area on the back of the mouse was the same as that in our earlier experiments.

Histologic and histoquantitative techniques.—The skin biopsy technique has been described (2-5). The samples were taken between 8 and 9 a.m. on the 2d, 6th, 10th, 16th, and 30th days after the first treatment. In experiment D (local application with MCA) the same procedure was followed but the first biopsy was taken on the 30th plus 2 days after the application. On each biopsy day, samples were taken from 5 different mice in each series. Specimens for electron microscopic examination were prepared at the same time (report to be published later). The control series comprised 100 untreated animals.

The specimens were fixed in Susa mixture, embedded in paraffin, and stained with hemalum and eosin. The cell count, which has been previously described, was made from the interfollicular area proper; the vicinity of the follicular openings was carefully avoided. Cells in the stratum malpighii were counted from 15 fields per specimen. Every field was 90 μ long so that the whole length, accordingly, was 1350 μ —microscopic magnification \times 1,440 (oil immersion). The following cell types were differentiated: (1) basal cells, (2) differentiating cells, (3) degenerating cells, and (4) mitotic cells.

RESULTS

Normal Interfollicular Epidermis of the Back of the Mouse

The interfollicular epidermis (IFE) of the back of the male mouse is composed of 1 or, at the most, 2 layers of cells surmounted by a relatively abruptly appearing narrow band of keratin (fig. 1). Most of the cells are in contact with and adhere to the dermo-epidermal junction, which appears as a slightly undulating line. The cytoplasm is basophilic, the nuclei are round or oval, and mitotic figures appear only in scattered cells. The intercellular spaces cannot be visualized under the light microscope. In the flattened superficial cells, small amounts of keratohyaline granules stained with hemalum and eosin. The microscopic appearance of the IFE was uniform in all the 100 control animals [for the structure of dermis and cutaneous appendages see (2, 4, 5)].

Histoquantitative analyses revealed that in the counted area the mean total number of cells was 243 ± 2.2 , excluding stratum granulosum. They consisted of 172 ± 1.9 (71.1 \pm 0.46%) basal cells, 61.7 ± 1.6 (25.4 \pm 0.46%) differentiating cells, 8.44 ± 0.65 (3.48 \pm 0.27%) degenerating cells, and 0.15 (about 0.06 \pm 0.02%) mitotic cells. The mitoses were located exclusively in the basal-cell layer.

Experiment A: Single Application of 60 γ of MCA and of 60 γ of DMBA

Since the time of appearance of changes in the IFE depended on the product used for treatment, the observations are divided into stages depending on the elapsed time after the first treatment.

On the 2d day (fig. 2) the IFE was 2 to 3 times thicker than normal, but the number of cells had not increased though they were greatly increased in size; the nuclei were enlarged, and the cytoplasm exhibited swelling, vacuolation, and pallor. The nuclear size varied considerably and many nuclei were karyolytic, while the chromatin was irregularly distributed, frequently along the nuclear membrane. Distinct intercellular bridges had developed with wide intercellular spaces. The amount of keratohyaline granules had increased here and there. On the 6th day (fig. 4) the cell proliferation in the IFE had increased and the cells were somewhat more regular in form. The basal-cell layer could be distinguished from the prickle-cell layer, which had larger cells. A few nuclei were pyknotic. The nuclear size and the amount of chromatin varied from cell to cell. Mitotic figures, in addition to those appearing in the basal-cell layer, were scattered in other layers as well. Stratum granulosum was uneven. In all animals the IFE was transformed into a hyperplastic and hyperkeratotic state, though the hyperkeratosis was irregular. From this day the IFE was markedly thicker and the total number of cells was greater in the DMBA than in the MCA series. By the 10th day the epidermal hyperplasia had distinctly increased in the DMBA series. In the MCA series the degree of hyperplasia showed no clear difference when compared with the status on the 6th day. In

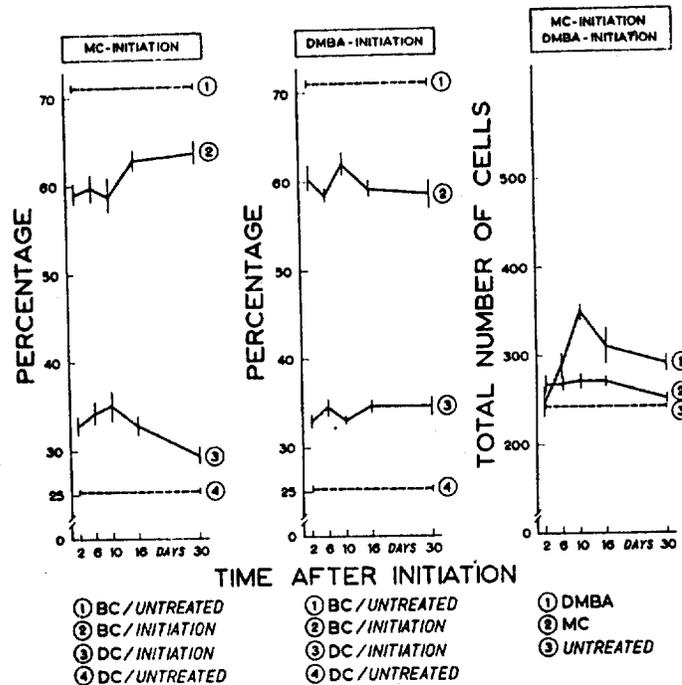
general, the changes in the DMBA and MCA series were reduced by the 10th day: The cells were smaller, and the nuclei uniform and more darkly stained, but the stratum granulosum and stratum corneum were thick. On the 16th day the changes in both series had decreased: In the MCA series the cell size had nearly reverted to normal; stratum granulosum was only present here and there. The DMBA series was clearly different: All layers in the IFE could still be differentiated. On the 30th day (figs. 3 and 5) a further decrease in the changes was seen. However, animals that received this application of DMBA and MCA clearly differed from the controls: Stratum corneum was essentially thicker than that in controls but stratum granulosum was hardly detectable. The cells in stratum malpighii were small, with poorly defined boundaries. Although the chromatin took a strong stain, the amount was variable in different nuclei.

Typical of the DMBA and MCA series were destructive changes in the follicles, which were present on the 2d day. The hair sheaths were hyperplastic, and keratotic plugs were seen in the dilated follicular openings. The sebaceous glands had almost completely disappeared by the 6th and 10th days, and numerous follicles were epilated. Alterations in the pilosebaceous apparatus were clearly more severe in the DMBA series. This was also true at the later experimental stage, though the changes in the pilosebaceous apparatus recovered with time. On the 30th day the openings of only a few follicles were dilated and keratinized, and the sebaceous glands began to regain a more normal appearance. The dermis showed an inflammatory reaction and this also decreased with time.

Histoquantitative analyses (text-fig. 1) revealed, on the 2d day, a nearly normal total cell number: MCA, 268 ± 13 , and DMBA, 248 ± 20 . On the 6th day the total number of cells in the DMBA series had increased; it was highest (350 ± 11) on the 10th day and decreased thereafter. In the MCA series, again, the total cell number had increased only slightly—on the 30th day it was nearly normal (252 ± 6.9). The number of differentiating cells was relatively higher than that of the basal cells, and the amount of degenerating cells varied considerably.

Experiment B: Continuous Treatment (6 Times a Week) With 60 and 0.06 γ of MCA and DMBA

Carcinogen per dose, 60 γ .—On the 2d day both series (MCA and DMBA) showed a large increase in the size of cells and nuclei, but with variations in size, especially in the DMBA series. Intercellular spaces had formed which were irregular; the nuclei were vesicular and pale. Distribution of chromatin was irregular, frequently along the nuclear border. Abundant karyolysis was also present. By the 6th day (fig. 6) a relatively high-degree IFE hyperplasia had developed. Its various cellular layers were distinct though irregular in form. Nuclear size varied greatly, but chromatin distribution was more regular. No karyolysis was seen. In some places most cells were a basal type; there were moderate amounts of mitotic cells. On the 10th and 16th days marked disorganization was



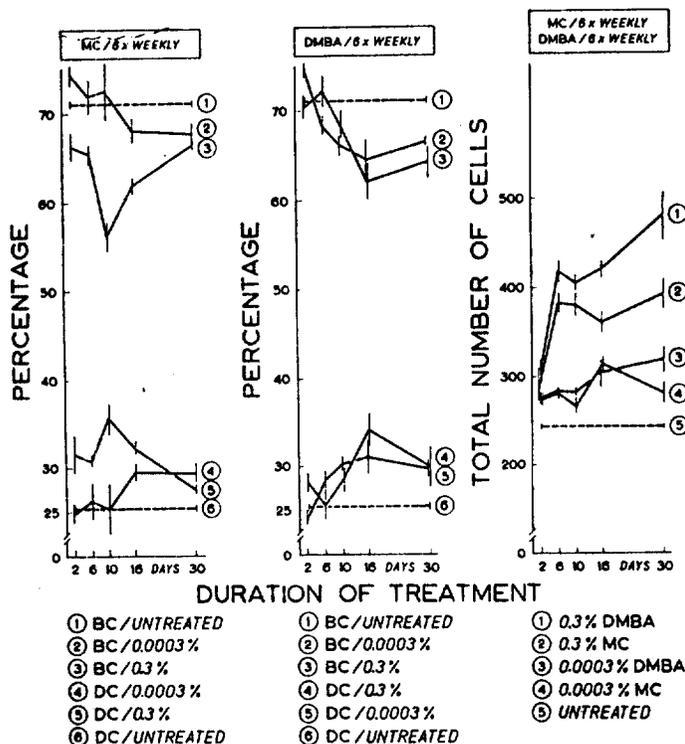
TEXT-FIGURE 1.—Development and degree of hyperplasia (alterations in the total cell count), and percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: single application ("initiation") of 60 γ MCA and of 60 γ DMBA. (Vertical lines: mean \pm S. D. of the mean.)

conspicuous particularly in the DMBA series. Cellular irregularities (fig. 8) were considerable in both series. There were, in some cells, completely atypical, very large, and/or irregularly shaped nuclei. Pathologic mitoses were also present. Intercellular spaces were wide and irregular. In the DMBA series, especially, the IFE was again difficult to distinguish from the enlarged and greatly hyperplastic follicles. On the 30th day, with DMBA (fig. 7), the amount of irregular hyperplasia had increased further. The altered IFE formed, with the cells of follicular origin, large, irregular extensions and vegetation downward into the dermis. The cells in these extensions showed a disorderly arrangement; there also was moderate atypia and, in some places, dyskeratosis. In the MCA series, also (fig. 9), the cell damage and general irregularity of the IFE were accentuated, but not to the same extent as in the DMBA series. The thickness of the stratum granulosum varied considerably. Here and there the epidermal surface was parakeratotic. The stratum corneum was usually abundant.

As the treatment period progressed, the follicles exhibited progressively increasing destructive alterations that were connected with follicular hyperplasia. From the 6th day nearly all the sebaceous glands had disappeared or they appeared as deformed remnants. The follicular openings were dilated, increasingly filled with keratin plugs, and, in most instances,

totally epilated. On the 30th day the deformed follicles in many places in the corium formed irregular cysts filled with horny material. From the beginning there were marked inflammatory alterations throughout the corium. Later the upper corium, especially, appeared rather degenerated—it was edematous, somewhat homogenized, and had few cells. The changes in the lower corium seemed more exclusively inflammatory.

Histoquantitative analyses (text-fig. 2) revealed that on the 2d day the total number of cells had slightly increased from normal: MCA, 291 ± 18 , and DMBA, 307 ± 9.6 . But from the 6th day the cell count disclosed a



TEXT-FIGURE 2.—Development and degree of hyperplasia (alterations in the total cell count), and percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: 60γ (as 0.3% acetone solution), and 0.06γ (as 0.0003% acetone solution) of MCA and DMBA, 6 times a week.

high-degree hyperplasia: MCA, 382 ± 12 , and DMBA, 419 ± 14 . Both the absolute number of basal cells and that of differentiating cells had greatly increased. Their numerical ratio therefore differed very little from that in the normal IFE. The percentage of degenerating cells was rather high; *e.g.*, on the 30th day: MCA, 5.9 ± 0.47 , DMBA, 5.6 ± 0.93 . The percentage of mitotic cells was highest in the MCA series on the 10th day ($0.6 \pm 0.1\%$) and in the DMBA series on the 6th day ($0.5 \pm 0.1\%$). Mitotic figures, in addition to those appearing in the basal-cell layer, were scattered in other layers also. On the 30th day the total number of cells

in the MCA series was 392 ± 18 and in the DMBA series, 480 ± 28 . The degree of hyperplasia had progressively increased.

Carcinogen per dose, 0.06 γ .—On the 2d day the IFE showed, in some places, a definite though low-degree increase in cell size. On the 6th day (figs. 10 and 12) there were areas in the IFE in which the cells were much above normal in size; the total number of cells, however, was nearly unchanged. The intercellular spaces in these areas were distinct. The basal- and prickle-cell layers were distinguishable and developed like the stratum spinosum and the thin stratum granulosum; the stratum corneum was thicker than normal. The nuclei stained intensely. Differences were observed between the two series in that the increase in cell size and the general increase in the IFE thickness were greater in degree in the DMBA series. Typical of both series were regional variations in the intensity of the alterations. On the 10th day the situation was similar to that on the 6th day. On the 16th day the IFE was hyperplastic here and there. On the 30th day (figs. 11 and 13) the variations in size and staining of nuclei had increased further, though cells generally were relatively small. In many places there was hyperkeratosis.

The follicles exhibited comparatively few changes, which appeared chiefly on the 16th day. Some follicular openings had keratin plugs. In the upper corium there were only slight inflammatory reactions.

Histoquantitative analyses (text-fig. 2) revealed that on the 2d day the total number of cells had only slightly increased from normal: MCA, 274 ± 7.3 , and DMBA, 277 ± 7.3 . On the 16th day the hyperplasia was marked: MCA, 315 ± 11 , and DMBA, 305 ± 17 . The percentage of mitoses was manifold compared with the normal: MCA, 0.4 ± 0.2 , and DMBA, 0.4 ± 0.2 , on the 30th day. Mitotic figures, in addition to those in the basal-cell layer, were sometimes observed in more superficial layers. With these low doses of carcinogen, the difference in potency between MCA and DMBA is not so clear.

Experiment C: Continuous Local Treatment (6 and/or 12 Times a Week) With t. and s. Tween 60, s. Span 60, and s. Tween 20, Without Preceding Carcinogen Initiation

Changes in IFE produced by other technical products of this group have been reported earlier (2, 4, 5). For this reason the experiment was repeated only with the most potent compound, t. Tween 60. The effect of Span 20, the parent compound of Tween 20, is analyzed in paper II (16).

T. Tween 60 and s. Tween 60.—These two compounds had a similar effect on the IFE. On the 2d day the IFE had very greatly thickened; the number of cells increased and they were arranged in 3 or 4 layers. Cells and their nuclei were large and stained lightly—nuclei were vesicular, and chromatin was regularly arranged. Many cells showed perinuclear vacuolation, which was greatest in the series treated 12 times a week (2 times a day) with s. Tween 60. The intercellular spaces were wide, and they as well as the well-differentiated intercellular bridges were uniform. Some of the basal cells were of a spinous type. Parakeratosis was present

especially in the series treated 12 times a week. On the 6th day the number of cells showed further increase. In general the alterations were similar, but the different cell layers were more distinctly discerned. The nuclei of the basal cells were more intensely stained than those of the prickle-cell type and they had less cytoplasm. In some of the specimens the stratum granulosum contained keratohyaline granules, and in some there was parakeratosis. Intracellular vacuolation was seen chiefly in the series treated 12 times a week. On the 10th, 16th, and 30th days very little of this remained, but the intercellular spaces continued to be wide. The thickness of the stratum granulosum and stratum corneum was related to the amount of hyperplasia, though many specimens still showed parakeratosis (figs. 14 and 15). The size of the cells and nuclei varied comparatively little through the period of experiment. No pathologic mitosis was seen. There were occasional pyknotic nuclei, but other damage to cells or nuclei was not seen.

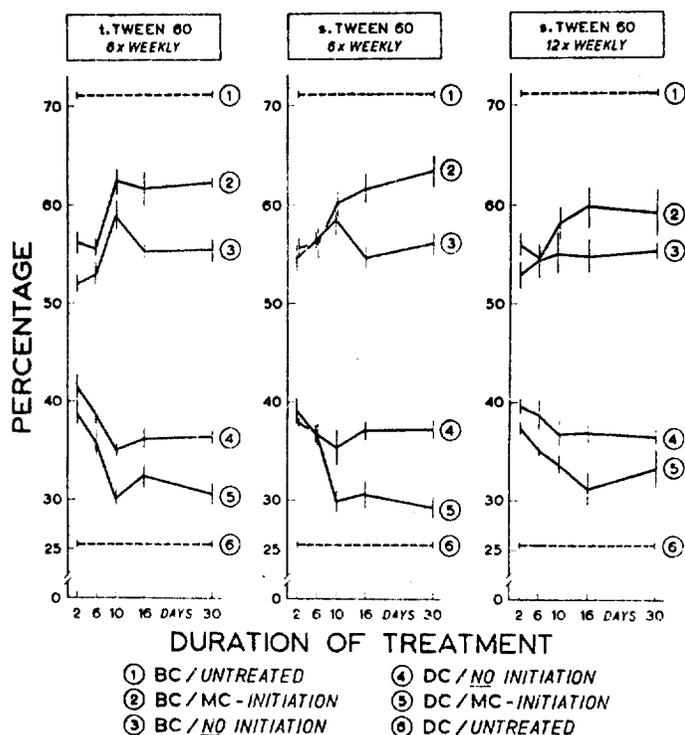
In many instances the hyperplasia of the IFE extended to the upper portion of the follicles; the follicular openings were then slightly dilated and keratinized. The general shape of the follicles nevertheless was regular, and the hair was intact. The follicles were slightly larger than normal, independent of the phase of hair growth, and more than half the follicles were in the growth phase (*cf.* 2, 4, 5). From the 2d day the dermis was edematous, and throughout the experimental period there were diffusely distributed inflammatory cells.

Histoquantitative analyses (text-figs. 3 and 5) revealed that on the 2d day the total number of cells had greatly increased in all series but most in the s. Tween 60 (6 times a week): 399 ± 8.0 . On the 6th day the thickening of the IFE was still more marked. Thereafter the total number of cells remained at approximately the same level in all series other than that treated 12 times a week in which it had increased further; the total number of cells was highest on the 30th day: 484 ± 26 . The hyperplastic IFE was composed chiefly of differentiating cells, whereas the basal cells had hardly increased. The percentage of degenerating cells was fairly constant (from 5 to 7%). The percentage of mitotic cells increased throughout the experimental period, but was highest on the 2d day: t. Tween 60, 1.7 ± 0.2 , and s. Tween 60, 1.3 ± 0.3 percent. Mitotic figures appeared only in the basal-cell layer, and were never observed in other layers of the hyperplastic IFE.

S. Span 60 and s. Tween 20.—On the 2d day, in the s. Span 60 series, a slight thickening was observed in the IFE. Cells and nuclei, however, were fairly normal in size or slightly larger. In the two series treated with s. Tween 20 (treatment 6 and 12 times a week) the IFE had thickened, and the cells and their nuclei had increased in size; there were narrow intercellular spaces and occasional intercellular bridges were visible. A narrow stratum granulosum was also discerned. On the 6th day the IFE of a few animals treated with s. Tween 20 was clearly hyperplastic. In the s. Span 60 series there was no change from the situation of the 2d day. On the 10th day the IFE in the s. Tween 20 (12 times a week) series

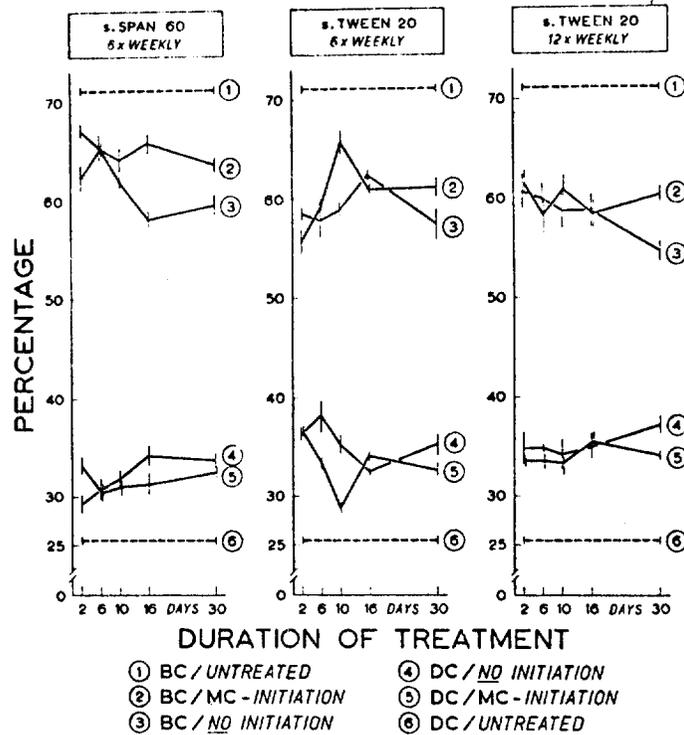
was still slightly hyperplastic, but in the series treated 6 times a week, the epidermal reaction had become weaker and was approximately similar to that in the s. Span 60 series. In the latter, the cells were relatively small, but in the s. Tween 20 series they were slightly larger. On the 16th and 30th days (figs. 18 and 20) the appearance of the IFE was practically unchanged.

Histoquantitative analyses (text-figs. 4 and 5) revealed that the total number of cells in the IFE had not increased to any noteworthy degree in the s. Span 60 series during the entire treatment period. In the two s.



TEXT-FIGURE 3.—Percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: t. and s. Tween 60 with and without initial MCA application.

Tween 20 series, the total number of cells had definitely increased on the 2d and 6th days. Thereafter the number of cells in the s. Tween 20 (12 times a week) series increased throughout the experimental period; and in the s. Tween 20 (6 times a week) series the total cell number declined to nearly normal. Despite the small change in the total number of cells, there was a larger number of differentiating cells than in the normal IFE. The percentage of degenerating cells was below normal. Mitotic cells had increased in number, which was greatest in the s. Tween 20 series treated 12 times a week. The number of mitotic cells was not nearly as high in any of these series as in the t. and s. Tween 60.



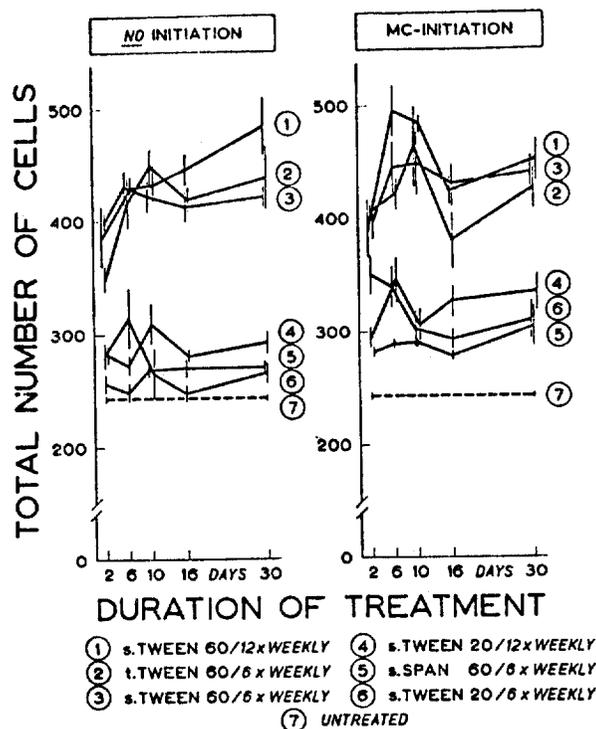
TEXT-FIGURE 4.—Percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: s. Span 60 and s. Tween 20 with and without initial MCA application.

Experiment D: Continuous Local Treatment (6 and/or 12 Times a Week) With t. and s. Tween 60, s. Span 60, and s. Tween 20, After Initial Local Application of 60 γ of MCA

T. and s. Tween 60.—The effect of these two compounds on the IFE, pilosebaceous apparatus, and dermis was similar.

The histologic appearance differed completely from that found to be characteristic of the effect of these agents when used without an initial carcinogen application (expt. C).

When a secondary treatment with t. and s. Tween 60 was begun 30 days after an application of MCA, the IFE, on the 2d day, was hyperplastic to a high degree and consisted of 3 to 4 layers of cells. The cells and their nuclei had increased in size, and there was cellular and nuclear atypia. Cytoplasm and nuclei stained more intensely and were more basophilic than in the series without an initial application. Intracellular vacuolation was not seen; the intercellular spaces were wide and the intercellular bridges distinct; parakeratosis appeared occasionally; and the diameter of stratum granulosum varied. On the 6th day the thickness of the cell layers had increased further. The comparatively small, intensely stained basal cells now formed several layers here and there, with only a small number of larger-size prickle cells situated above. In some places



TEXT-FIGURE 5.—Development and degree of hyperplasia (alterations in the total cell count) in IFE. Treatment: t. Tween 60, s. Span 60, s. Tween 60, and s. Tween 20 with and without initial MCA initiation.

there was hypergranulosis and hyperkeratosis, in others there were areas in which the changes resembled those produced by these compounds alone; parakeratosis was uneven. In some cells there was also intracellular vacuolation, though very slight in amount. Through the 10th, 16th, and 30th days the degree of hyperplasia remained nearly unchanged. Edematous changes, however, had decreased. In extensive areas of the IFE, basal cells predominated (figs. 16 and 17).

In these series the follicles also exhibited various alterations. The IFE hyperplasia extended fairly deep; the openings of numerous follicles had keratin plugs; and part of the follicles were epilated. The dermis had thickened and showed mild inflammatory changes.

Histoquantitative analyses (text-figs. 3 and 5) revealed that in all series there was a high-degree increase in the total cell number. This was highest on the 6th and 10th days after secondary treatment had begun. However, the total cell number was at the same level as in the series without an initial application of MCA. Cell counting showed, in all the MCA series, a great increase in the number and percentage of basal-type cells compared with the series treated with t. and s. Tween 60 alone. The percentage of mitotic cells in the noninitiated series (expt. C) was somewhat higher: mean, 0.93 percent, while in the series treated with the same agents after initiation, the mean was 0.78 percent. In contrast to those

series mentioned, which were treated without an initial application of carcinogen, mitotic figures *now* appeared also in the more superficial layers of the hyperplastic IFE. The percentage of degenerating cells in the MCA series varied from 4 to 10 percent.

S. Span 60 and s. Tween 20.—In contrast to the preceding, the changes differed comparatively little from those seen in the series without MCA (expt. C). In the *s. Span 60* series, only scanty changes were found in IFE compared with those present before the secondary treatment. The thickness of IFE had only slightly increased. The cellular size was nearly normal (*cf.* figs. 18 and 19). In the *s. Tween 20* series there was a low-degree IFE hyperplasia by the 2d day, and the cells had clearly increased in size. Intercellular spaces, although narrow, were visible. They persisted practically unchanged to the 6th, 10th, and 16th days and, partially, to the 30th day. The stratum corneum was thicker than normal throughout this time. In contrast to the noninitiated series, the changes by the 30th day varied from area to area, *i.e.*, among others, the size and staining of nuclei and the thickness of stratum corneum (*cf.* figs. 20 and 21).

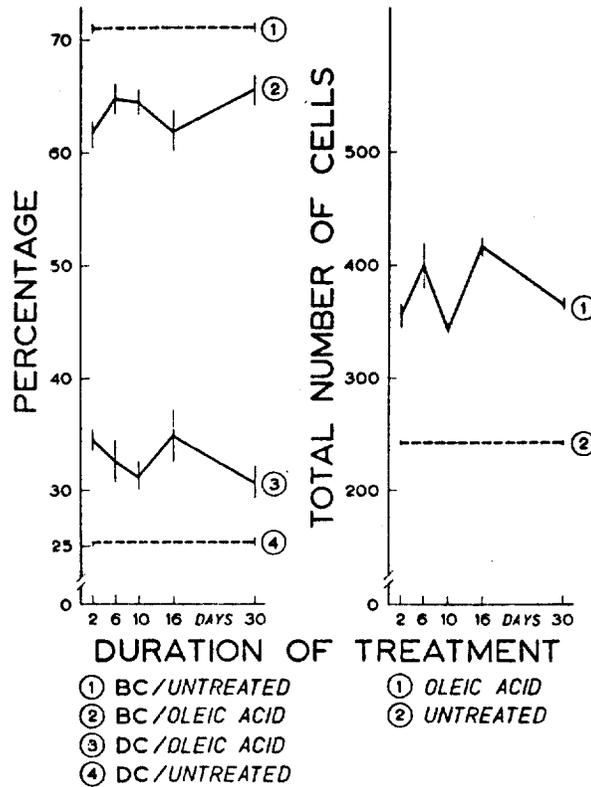
The follicular openings were in some places dilated and contained some keratin plugs. The dermis was somewhat thickened and here and there contained inflammatory cells.

Histoquantitative analyses (text-figs. 4 and 5) revealed that the total cell number in the stratum malpighii had increased in all series and most in those given the secondary treatment 12 times a week with *s. Tween 20*. The total number of cells in the IFE was slightly higher than in the corresponding noninitiated series. The percentage of basal cells had greatly increased in the initiated series, though differences were slight. There was no difference in the percentage of mitotic or degenerating cells.

Experiment E: Continuous Local Treatment (6 Times a Week) With Undiluted Oleic Acid Without Initial Carcinogen Application

Certain general features of the skin changes produced by this fatty acid after carcinogen application have been described (17). The nature of the hyperplasia produced by undiluted oleic acid alone will be analyzed here. On the 2d day the IFE was hyperplastic, the cells were greatly enlarged, and in some places the cytoplasm was swollen and pale. The nuclei had increased in size, and chromatin was normally distributed within the nuclei. Mitotic figures, moderately present, were normal in structure. Intercellular spaces had formed, and intercellular bridges were well differentiated. In some places there was parakeratosis, in others the stratum granulosum and stratum corneum were regularly formed. On the 6th day the degree of hyperplasia had somewhat increased. Cell layers were regularly formed, basal cells were smaller than prickly cells, and the nuclei stained uniformly. On the 10th (fig. 22), 16th, and 30th days (fig. 23) the degree of hyperplasia was approximately as before, only some variation in thickness of the IFE was seen from animal to animal. In some mice the stratum granulosum was remarkably thick.

had increased greatly from normal, and the percentage of mitotic cells was moderately high: 0.8 ± 0.1 percent, on the 2d day. The mitoses were located exclusively in the basal-cell layer of the hyperplastic IFE. There were few degenerating cells.



TEXT-FIGURE 6.—Development and degree of hyperplasia (alterations in the total cell count), and percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: undiluted oleic acid, without initial carcinogen application 6 times a week.

DISCUSSION

The primary changes produced by agents that influence skin-tumor formation may be of a different type than the changes that develop later. The alterations may be transitory, permanent, or progressive. For evaluation of the nature and significance of the cutaneous changes, it is

definitely necessary to observe their development *over a long period*. The *nature* of epidermal hyperplasia must be studied to derive adequate results.

The IFE of the back of the mouse is an excellent target tissue. It is composed of one—at the most two—layers of cells, and most cells are undifferentiated (4, 5). The mice of the strain used in this laboratory are well suited for the purpose, since their skin has no features that may be correlated to tumor formation. The changes produced by each test compound were, in general, the same in all animals.

It has been reported previously (9) that products of Span 60 (sorbitan monostearate), Tween 60 (polyoxyethylene sorbitan monostearate), Span 20 (sorbitan monolaurate), and Tween 20 (polyoxyethylene sorbitan monolaurate), which we synthesized under controlled conditions, resembled the corresponding technical products in their tumor-promoting property or lack of it.

It has now been demonstrated that the synthesized products also produce fully related cutaneous changes. The "Tween 60 effect" (Tween 60 was obtained by adding about 20 ethylene oxide units to inactive s. Span 60) is not a result of by-products of the synthesis. Span 20, which contains no ethylene oxide chains, has, in itself, a moderate tumor-promoting property (6). But when a product of the type of Tween 20 is prepared from it, the tumor-promoting property, as well as the hyperplasia-causing effect of the resulting product, decline.

The effect of a locally applied compound of the Tween 60 type completely differs from the skin changes produced by carcinogens applied in the same manner, *i.e.*, MCA and DMBA. These differences are demonstrable also by a rough, light-microscopic technique, and the following are stressed:

1. A single application of 60 γ of carcinogen produced, immediately, skin changes of a toxic nature, including severe cell and nuclear damage. Reparative cell proliferation began only after these changes had become quiescent. Similar treatment with a Tween 60-type product did not cause retrogressive changes; intense cell proliferation began immediately in the IFE. The same was true of a high-degree hyperplasia (text-figs. 1 and 5).

2. Continuous treatment with a carcinogen (60 and 0.06 γ , respectively, 6 times a week) produced changes in the IFE that were greater, more effective, and/or longer than the duration of the action of the carcinogen. A severe degree of cell atypia developed and abnormal nuclei and progressive disturbances in the organization and differentiation of the cells. The percentage of compactly built basal-type cells increased progressively, with a relative decrease in differentiating cells. The percentage of mitotic figures increased considerably. Pathologic mitoses were abundant. Mitotic figures were also observed in the more superficial layers of the hyperplastic IFE. Hyperplasia was absent on the 2d day, but it developed thereafter and increased progressively throughout the treatment period (text-fig. 2). When the dose of carcinogen was low (0.06 γ) this progressive increase in proliferation was not manifested to any noteworthy

degree, though there were definite changes in cells and nuclei. Similar treatment with a Tween 60-type product (an effective tumor promoter) did not cause cell or nuclear atypia in the IFE, but the size of cells was clearly and uniformly increased. A strong intercellular edema developed, especially in the basal parts of the IFE, and well-differentiated intercellular bridges appeared. Further typical changes were a strong intracellular edema and perinuclear vacuoles, which had previously been found typical of the corresponding technical products (2, 4, 5). The percentage of differentiating cells increased evenly and greatly, with a relative decrease in basal cells. The number of mitoses was high, but no pathologic mitoses were seen. All mitoses appeared in the basal-cell layer. A high-degree hyperplasia developed immediately. Cell layers were uniformly and regularly arranged. The amount of hyperplasia increased up to the 16th day; treatment thereafter merely maintained it unchanged (text-figs. 3 and 5). The alterations did not change essentially when the applications were doubled (12 times a week). The slight but immediately appearing changes due to products of the Span 60 type (no tumor-promoting effect) and Tween 20 (weak tumor promoter) remained unchanged or were, at the end of the observation period, even less pronounced (Tween 20) than the first reaction had been (text-figs. 4 and 5).

3. A similar treatment with carcinogen, as mentioned in paragraph 2, and even the initial application alone, produced follicular destruction, epilation, partial or total disappearance of the sebaceous glands, and, in the root sheaths, changes similar to those in the IFE. Definite degenerative changes developed in the dermal components. Similar treatment with a Tween 60-type product produced mild hyperplasia in the pilosebaceous glands, which remained completely unchanged when the frequency of treatment was doubled. The dermis increased in thickness and exhibited edema and a uniform mild inflammation.

4. The nature and degree of skin changes that developed after a single initial application of carcinogen as well as during continuous treatment with carcinogen varied essentially even in different parts of the treated area. Changes produced by a Tween 60-type product were similar throughout the whole area.

5. Under experimental conditions, MCA was better suited for these studies than DMBA—MCA initiation produced a comparatively low-degree hyperplasia. The thickness of the IFE reverted to nearly normal in 30 days (text-fig. 1). Experiment D was specially constructed on the basis of this principle (*see* 6).

6. The cutaneous changes produced by continuous treatment (6 times a week) with a Tween 60-type product, which began after an interval of 30 days from the initial application of MCA, differed essentially from those produced by Tween 60 alone (*cf.* figs. 14 and 16). Cell and nuclear atypia developed. The cells of the IFE clearly exhibited increased resistance to the capacity of a Tween 60-type tumor promoter to produce intracellular edema (*cf.* 2). There was a marked tendency to keratinization; widened follicular openings had keratin plugs, etc. The percentage

of basal-cell-type elements increased significantly compared to differentiating cells (text-figs. 3 and 5). The effect of a single initial application of MCA was manifested not only in these structural changes but, above all, in a retardation and disturbance of the maturing of the IFE cells, when cell multiplication was forced to start *afterward* by secondary treatment, and was continuously maintained with Tween 60. Now mitotic figures were observed in several layers of the hyperplastic IFE. After MCA application, similar treatment with Span 60- and Tween 20-type products, which when used alone only produced a transitory weak hyperplastic reaction, did not manifest the alterations mentioned (figs. 18 to 21). Now the percentage of basal cells increased slightly (text-fig. 4) and the thickness of the IFE increased insignificantly (text-fig. 5).

7. Continuous treatment (6 times a week) with undiluted oleic acid alone (effective tumor promoter) produced persisting IFE hyperplasia (text-fig. 6) which, in principle, was the same as that caused by a Tween 60 product alone (*cf.* 2). Oleic acid, however, slightly affected the pilosebaceous apparatus. The general manner of reaction (figs. 22 and 23) was nevertheless essentially different from that evoked by the carcinogen.

It is characteristic of the action of dipole-type tumor promoters ["cocarcinogenic lipides" (18)] that their tumor-promoting property and capacity to produce a persisting epidermal hyperplasia are the more effective the more frequently the skin of a resistant-strain mouse is treated with the compounds, as such, or as aqueous solutions. We have previously stressed that a general benignity (2, 4, 5, 9) and a marked tendency to regression (5, 6) are characteristic of tumors promoted by Tween 60, and by the known tumor promoters of the most simple type such as certain fatty acids (17). This is true also of wound-healing as promoting noxa (*e.g.*, 19) and of croton oil (15). In some instances the sex of the mouse may be involved, *e.g.*, with Tween 60 (5, 12) and oleic acid (12, 17).

While seeking new tumor promoters for the mouse skin, by using the "frequent application technique" introduced in this laboratory (20), it has become evident that a complete spectrum of them exists, from highly potent to rather weak ones. Some of them are physiologic, which are formed and/or needed by the organism [certain fatty acids (12, 17), and glycerol fatty acid esters (21)]. These dipole-type, tumor-manifesting agents may be regarded as "true tumor promoters." Biologically it is immaterial whether the tumors result from the use of comparatively large total doses and/or from the use of frequent applications. The same high incidence of tumors can be obtained even with one tenth as large a total dose per animal, provided treatment is given often enough (5). The biological result in any case is the manifestation of tumors. Histologically they also fulfill all the criteria of tumors (4, 5). Accordingly there develops precisely the phenomenon for which the concept "tumor promotion" originally was created by Friedewald and Rous, 1944 (19).

Promotion of tumors in the skin of the back of the mouse can be

produced by numerous polyol fatty acid esters, by many fatty acids, and other dipole-type tumor promoters. In its purest form, tumor promotion caused by dipole-type agents is a process of a fully benign nature. Histologically and histoquantitatively, tumor promotion (in this case) is manifested as an intense and a continuous multiplication (proliferation) of epidermal cells, when the percentage of mitoses may increase tenfold and even 20-fold from the normal. As a result of this the amount of cells in differentiation increases. The condition should be regarded as comparable to "simple" reparative cellular proliferation. This activity merely provides the conditions for the manifestation of the actual carcinogenetic process started by the initiation or, in sensitive mouse strains, even without it. *The tumor-promotion process as caused by dipole-type agents is not a part of the carcinogenetic process itself* [cf. also data and discussion, e.g., in (2, 4, 5)].

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PLATE 86

All photomicrographs. Hemalum and eosin. $\times 530$

FIGURE 1.—Mouse #3086. Normal IFE of back of adult mouse: Cells in 1 to 2 layers, nuclei oval, cell margins indistinct, no intercellular spaces, keratohyaline granules as a scarcely visible band, and thin keratin lamellae at surface.

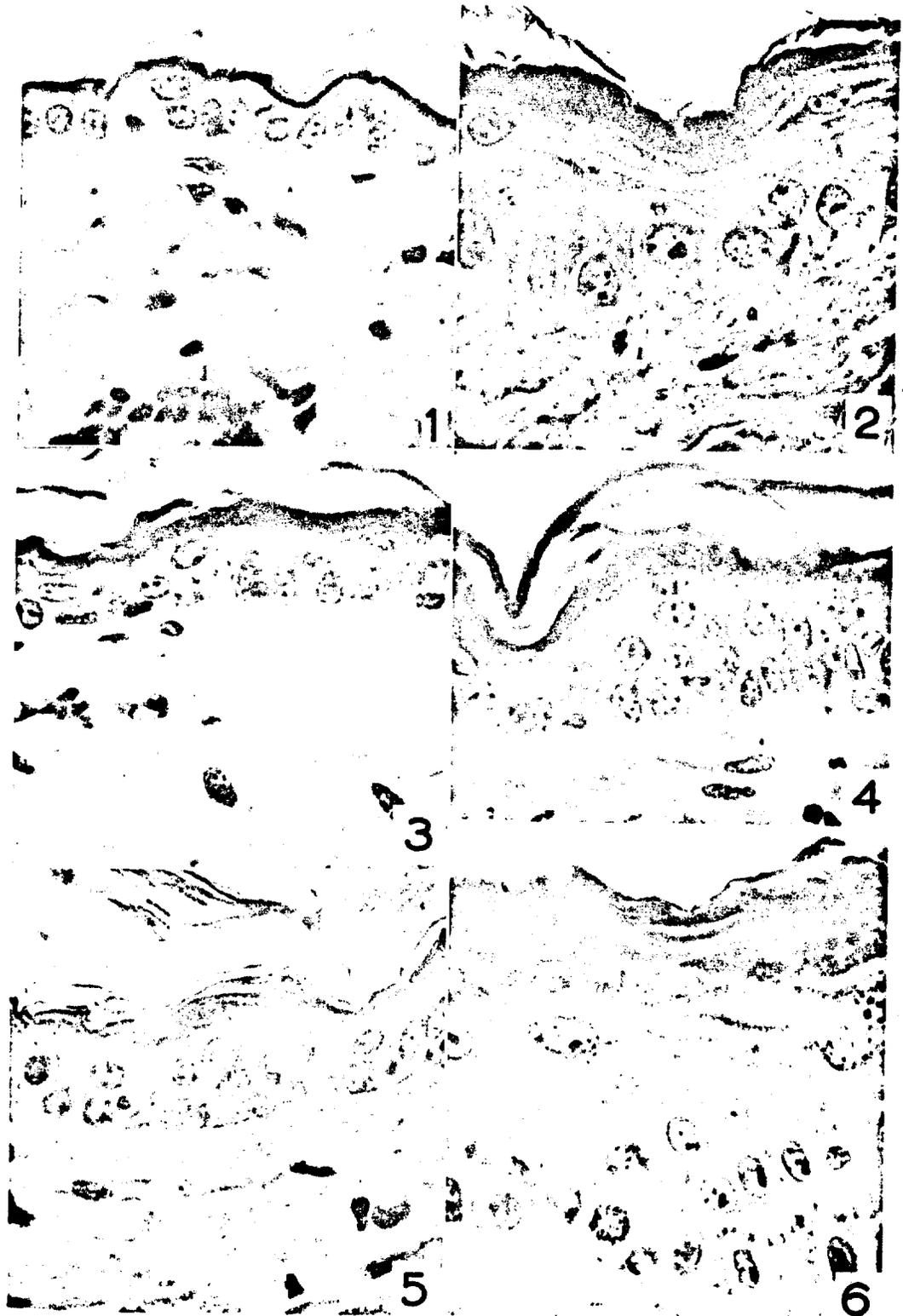
FIGURE 2.—Mouse #7135. A single initial application of 60 γ of DMBA; no secondary treatment, 2d day: IFE thickened, cells markedly increased in size, intercellular spaces wide and intercellular bridges distinct, nucleus/protoplasm ratio altered, and several enlarged nuclei.

FIGURE 3.—Mouse #7157. A single initial application of 60 γ of DMBA; no secondary treatment, 30th day: Thickness of IFE increased, cells and nuclei rather small, slight irregularity in nuclei, and stratum corneum thicker than normal.

FIGURE 4.—Mouse #7114. A single initial application of 60 γ of MCA; no secondary treatment, 6th day: Low-degree hyperplastic reaction, intercellular spaces narrow, size of nuclei slightly variable, increased chromatin content and increased keratinization.

FIGURE 5.—Mouse #7132. A single application of 60 γ of MCA; no secondary treatment, 30th day: Same alterations as in figure 4, but less pronounced.

FIGURE 6.—Mouse #9111, 60 γ of DMBA, 6 times a week, 6th day: IFE highly hyperplastic, cells irregularly increased in size, intercellular spaces wide and intercellular bridges visible, size and chromatin content of nuclei variable, and stratum granulosum and stratum corneum thick and irregular.



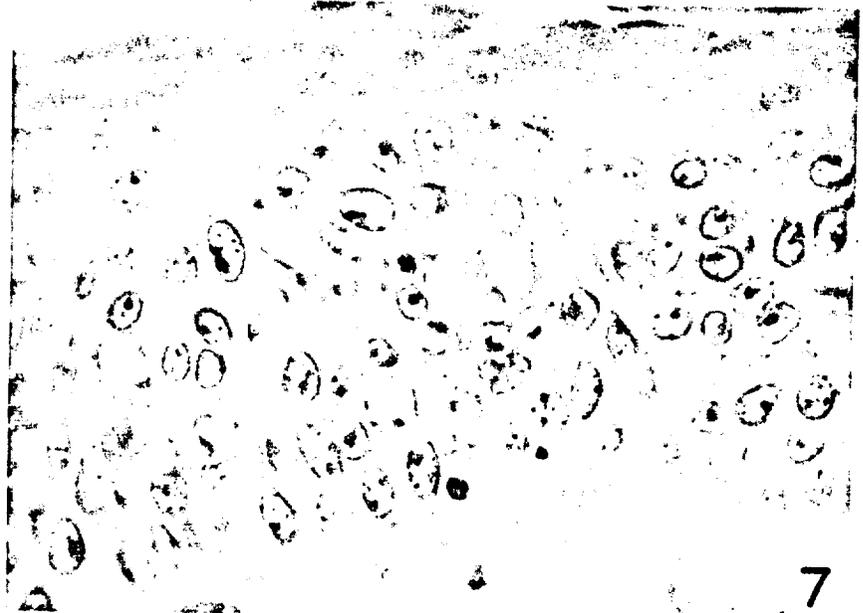
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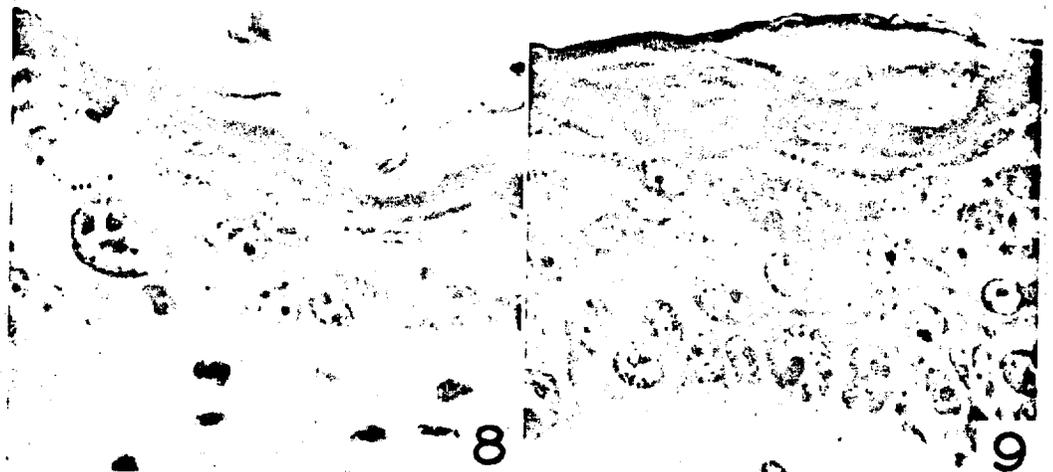
945

PLATE 87

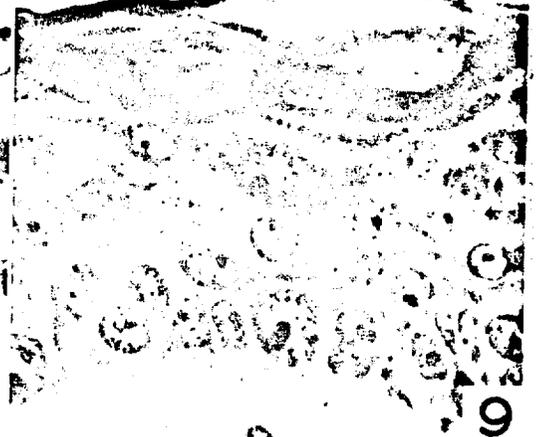
- FIGURE 7.—Mouse #9129, 60 γ of DMBA, 6 times a week, 30th day: Extremely high-degree hyperplastic reaction in IFE. cells and nuclei irregular in shape and variable in size, chromatin irregularly localized, nucleoles of different sizes, karyolysis, huge cells, and irregular intercellular bridge formation.
- FIGURE 8.—Mouse #9097, 60 γ of MCA, 6 times a week, 16th day: Irregular hyperplasia, marked variations in size of nuclei and cells, and also considerable atypia. (On *left*, a huge nucleus.)
- FIGURE 9.—Mouse #9101, 60 γ of MCA, 6 times a week, 30th day: High-degree and irregular IFE hyperplasia; nuclei and cells show great variation.
- FIGURE 10.—Mouse #9063, 0.06 γ of DMBA, 6 times a week, 6th day: IFE thicker than normal; size and chromatin content of nuclei variable.
- FIGURE 11.—Mouse #9078, 0.06 γ of DMBA, 6 times a week, 30th day: Slightly hyperplastic IFE thicker than normal, variation in nuclei and cells, and hyperkeratosis.



7



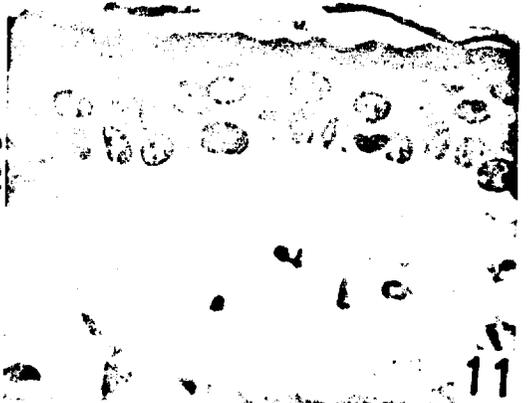
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PLATE 88

FIGURE 12.—Mouse #9036, 0.06 γ of MCA, 6 times a week, 6th day: Changes similar to those in figure 10.

FIGURE 13.—Mouse #9051, 0.06 γ of MCA, 6 times a week, 30th day: Unevenness and irregularity of IFE clearly evident.

FIGURE 14.—Mouse #7207, s. Tween 60, 6 times a week, without preceding initiation, 30th day: IFE even, regular IFE hyperplasia; cells in neat layers, cells and nuclei uniformly increased in size; intercellular spaces wide and intercellular bridges well differentiated, basal cells situated in one row, and differentiating cells abundant (*cf.* figs. 7, 9, 11, and 13).

FIGURE 15.—Mouse #7229, s. Tween 60, 12 times a week, without preceding initiation, 30th day: High-degree, regular IFE hyperplasia. Changes similar to those in figure 14, but intercellular spaces are wide and some pyknotic nuclei are seen; parakeratosis.

FIGURE 16.—Mouse #7357, s. Tween 60, 6 times a week, beginning 30 days after a single initial application of MCA, 30th day: Pronounced, irregular hyperplastic response, nuclei stained dark; and are comparatively small; basal type elements predominate, and number of true prickle cells is small.

FIGURE 17.—Mouse #7366, s. Tween 60, 12 times a week, beginning 30 days after a single initial application of MCA, 30th day: The same as in figure 16, but intercellular spaces markedly widened. Most cells are of basal type with spindle-shaped nuclei (longitudinal axis of cells at right angles to dermo-epidermal junction).

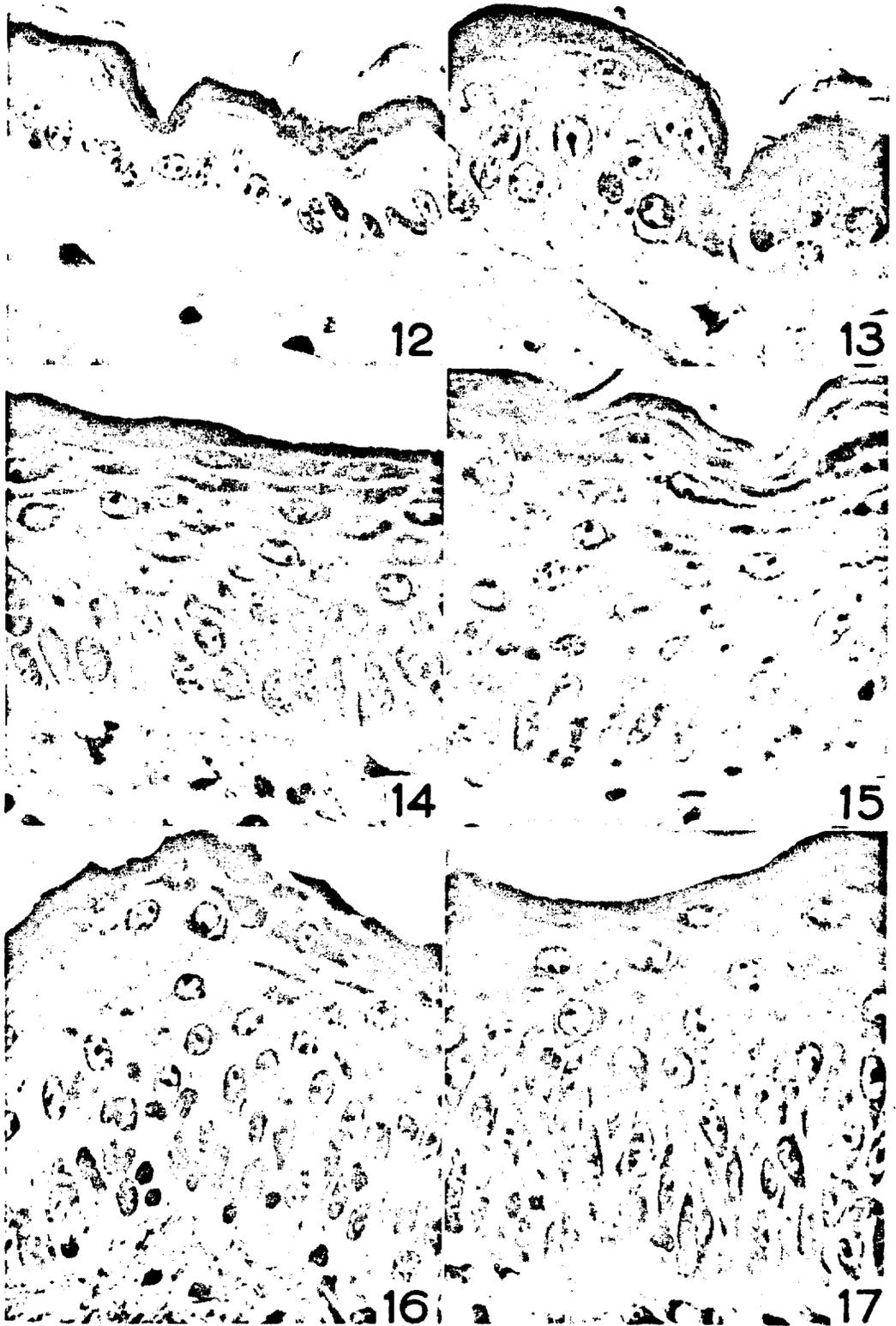


PLATE 89

FIGURE 18.—Mouse #7258, s. Span 60, 6 times a week, without initial carcinogen application, 30th day: IFE sparsely thickened; very small increase in number of cells (*cf.* fig. 14).

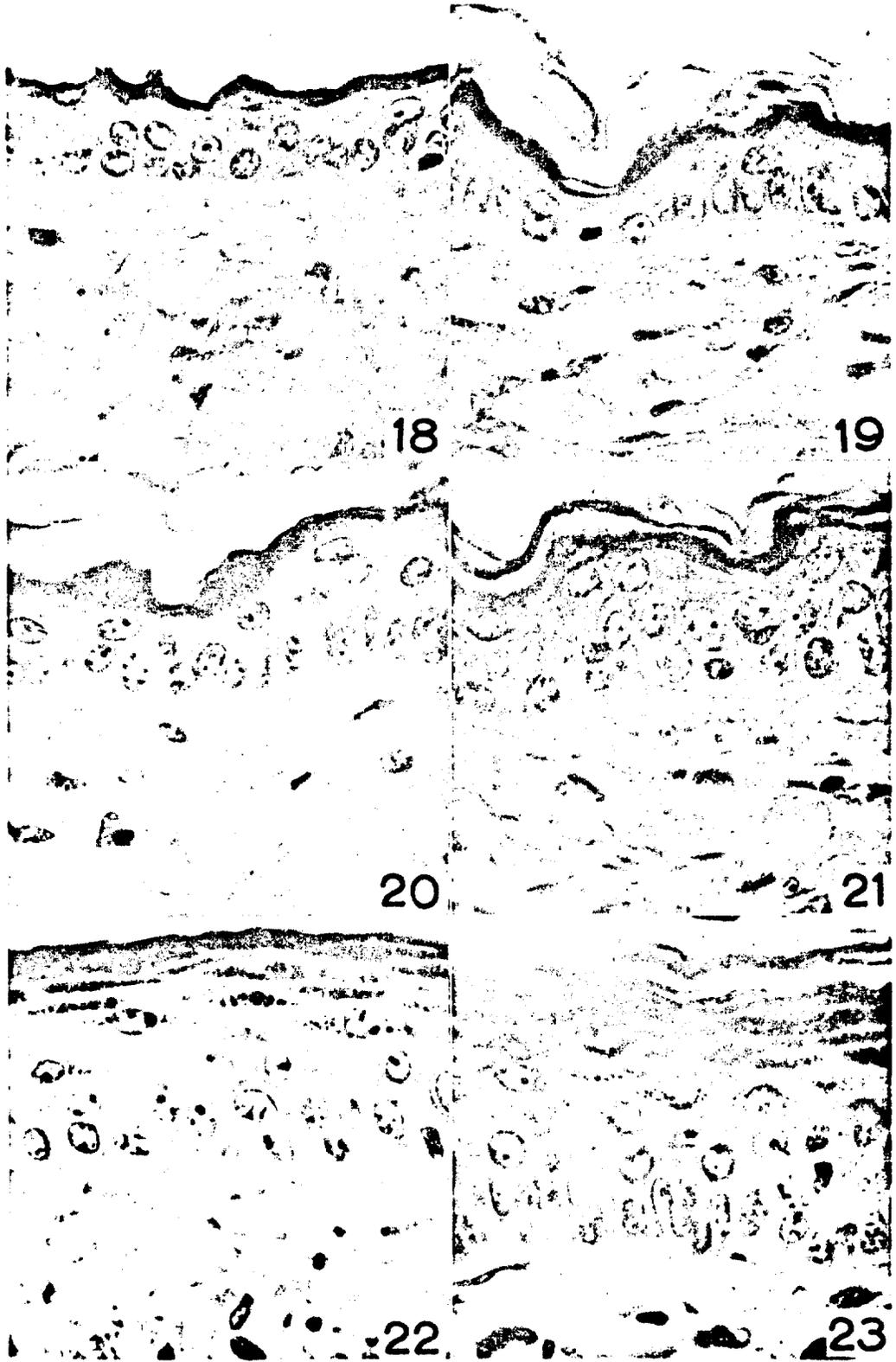
FIGURE 19.—Mouse #7409, s. Span 60, 6 times a week, beginning 30 days after a single initial MCA application, 30th day: Approximately same as figure 18, but possibly slight variation in size of nuclei; stratum corneum is thick.

FIGURE 20.—Mouse #7280, s. Tween 20, 6 times a week, without initial carcinogen application, 30th day: Low-degree IFE hyperplasia. Cells slightly but evenly increased in size.

FIGURE 21.—Mouse #7430, s. Tween 20, 6 times a week, beginning 30 days after a single initial MCA application, 30th day: Some small variations in nuclei; stratum corneum thickened (*cf.* fig. 20).

FIGURE 22.—Mouse #9016, oleic acid, 6 times a week, 10th day: Moderate IFE hyperplasia, cells and nuclei uniformly increased in size, cell layers even; intercellular, although narrow, spaces have formed; intercellular bridges well differentiated.

FIGURE 23.—Mouse #9028, oleic acid, 6 times a week, 30th day: Same as in figure 22, but basal cells situated in one layer slightly smaller. Compared with changes produced by s. Tween 60 (fig. 14) cells are slightly smaller, intercellular spaces narrower, and degree of IFE hyperplasia slightly lower.



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Studies on the Promoting Phase in the Stages of Carcinogenesis in Mice, Rats, Rabbits, and Guinea Pigs

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In previous experiments by Berenblum and Shubik (4), tumors were induced in mice by a single application of a carcinogenic hydrocarbon, followed by repeated applications of non-carcinogenic croton oil. Friedewald and Rous (7) similarly showed that tumors may be induced in the rabbit by a suboptimal dose of carcinogen followed by wound healing and applications of non-specific agents, such as turpentine. Previously, Berenblum (1) had shown that turpentine, as well as croton oil, had an augmenting (cocarcinogenic) effect in carcinogenesis in mice; and several other examples of this activity have been reviewed in the literature by Berenblum in 1944 and Rusch (15). Arising from this work, a new concept of carcinogenesis has been proposed, involving an initiating stage, brought about by the hydrocarbon, with a small number of normal cells changing into latent tumor cells which are subsequently converted into morphological tumors by promoting action (e.g., croton oil, wound healing, etc.). The latent tumor cells remain irreversibly changed for 20 weeks (4) or even as long as 43 weeks (5).

In the mouse, only croton oil can be said to have considerable promoting action, whereas other factors previously investigated, such as turpentine (1) and wound healing (6, 13) have a minimal effect. In the rabbit, on the other hand, both wound healing and turpentine seem to have a marked effect.

Substances tested for possible promoting activity have usually been selected from those agents known to induce epidermal hyperplasia. Mice and rabbits have most frequently been employed for such tests. In the present study, a variety of substances, all inducing epidermal hyperplasia, was examined for possible promoting activity, and, in addition to mice and rabbits, rats and guinea pigs were also used.

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METHODS

The mice used in this investigation were of the Swiss strain from the Medical Research Council, England, and were bred in this laboratory. Rabbits, rats, and guinea pigs were from the stock of the same laboratory. Throughout the investigation 9,10-dimethyl-1,2-benzanthracene as a 1.5 per cent solution in medicinal liquid paraffin (light mineral oil) was used as the initiating agent. Liquid paraffin was used as the solvent, except where solubilities made this impracticable. Test solutions were applied with a fine glass rod.

EXPERIMENTAL

Mice.— In this series, turpentine applications, already shown to have a mild cocarcinogenic effect in mice (1) and a noticeable action in rabbits (7), were reinvestigated as promoting agents after a single application of 1.5 per cent 9,10-dimethyl-1,2-benzanthracene. Next, a series of substances was examined in a similar way: (a) phenanthrene and fluorene, which are noncarcinogenic in mice (9, 10); (b) acridine, investigated by Kenaway (9) and Maisin *et al.* (11), and found to be noncarcinogenic but very irritating; (c) a series of vegetable oils, e.g., castor oil, its active principle ricinoleic acid, glyceryl monoricinoleate, and oleic acid, which had been shown (17) to augment carcinogenesis with inadequate dosage of 3,4-benzpyrene; (d) silver nitrate in aqueous solution; and (e) cauterization by electrocautery.

Each of these substances was first applied in serial dilutions to a small area of normal skin, in the interscapular region, which was clipped free of hair with scissors. After four applications, spaced over 2 weeks, the mice were killed, and pieces of tissue removed for histologic examination. The concentration yielding the maximum hyperplasia with the minimum of necrotic change was chosen for further testing. For the promotion tests, groups of ten mice were used, each receiving a single application of the carcinogen, and, after an interval

of 3 weeks, twice-weekly applications of the test solution. The turpentine was used as a 20 per cent solution, phenanthrene as a 1 per cent, acridine as a 0.3 per cent, and fluorene as a 0.5 per cent solution, all in liquid paraffin. Silver nitrate was made up as a 10 per cent aqueous solution. The remaining test substances were used undiluted. Finally, a group of five mice was cauterized with a superficial linear streak 1 inch long in the center of the painted area at monthly intervals following a single application of the carcinogen. In this group no tumors have been recorded at 60 weeks following the original application.

The results of the tests for promoting activity, summarized in Table 1, were uniformly negative.

TABLE 1
SUBSTANCES TESTED FOR TUMOR PROMOTION IN MICE

Substance tested	Skin histology ¹	Promotion test ²
20 per cent Turpentine in liquid paraffin	Minimal histologic change	No tumors recorded
0.3 per cent Acridine in liquid paraffin	Marked epidermal hyperplasia	"
0.5 per cent Fluorene in liquid paraffin	Slight epidermal hyperplasia	"
1 per cent Phenanthrene in liquid paraffin	"	"
Castor oil	"	"
Ricinoleic acid	"	"
Glyceryl monoricinoleate	"	"
Oleic acid	"	"
Silver nitrate (10 per cent aqueous)	Good hyperplasia, most marked in the hair follicles	"

¹ Groups of three mice; 4½ weekly applications.

² Groups of ten mice, given semiweekly applications for 20 weeks; 3 weeks after a single application of 9,10-dimethyl-1,2-benzanthracene in liquid paraffin.

RABBITS.—The experiments of Friedewald and Rous (7), showing that wound healing may act as a promoting agent in this species, were repeated but modified by using *only a single application* of carcinogen as initiator. Turpentine has been reinvestigated under these conditions, and the effects of croton oil studied in the rabbit. Similarly, chloroform and inorganic arsenic were reinvestigated under these conditions.

Berenblum (2) showed that 9,10-dimethyl-1,2-benzanthracene is a potent carcinogen for rabbit skin, and a single application of this hydrocarbon was therefore used as standard initiator for this part of the investigation also.

The effects of wound healing and turpentine.—Four areas of skin on the back of one rabbit, clipped free of hair with electric clippers, were used for the determination of the optimal dosage of turpentine; four strengths, undiluted, and 50, 20, and 10 per cent turpentine in liquid paraffin, were ap-

plied to these areas twice weekly. The undiluted turpentine induced ulceration within 1 week, and it was discontinued. The other three solutions induced macroscopic reddening and thickening of the skin. After 3 weeks the 50 per cent solution also caused some ulceration, and it, too, was discontinued; a skin biopsy was made for histological examination. The remaining concentrations were applied for up to 10 weeks, and the animal then killed. Histologically, extensive epidermal damage was noted in the skin treated with 50 per cent solution, and only minimal hyperplasia; with the 20 per cent solution there was considerably less damage, but, again, only minimal hyperplasia. The 10 per cent solution induced almost no change. In no case was an appearance produced resembling that described by Rous (14), where turpentine caused the epidermis to become from five to ten cells thick.

In spite of the unsatisfactory nature of the first test, but because of earlier favorable reports, the experiment was continued, using the 20 per cent solution, applied to three areas of skin on the backs of four rabbits, which had been treated 1 month previously with a single application of the carcinogen. The fourth area on each rabbit was maintained as a control for one application of carcinogen. These applications were continued twice weekly for 20 weeks; one rabbit died in the sixth week of the experiment and yielded no results. Concurrently, a hole $\frac{3}{4}$ inch in diameter was punched through the ears of each rabbit previously treated only once with carcinogen, using a sharp cork borer. Among the three surviving rabbits, one developed definite papillomas on both ears at the seventh week around the site of the punched hole, and another developed a papilloma on one ear at the eighth week following the trauma. All of these tumors continued to grow until the twentieth week when the animals were killed, but none became malignant. On the third rabbit tumors did not develop. There was no evidence of any tumor formation in any of the areas treated with turpentine.

The effects of croton oil on rabbit skin.—Again preliminary skin tests were carried out on one rabbit, with a 5 per cent solution of croton oil applied to two areas of skin and a 10 per cent solution to two others, at twice-weekly intervals for 2 weeks. Both solutions were made up in liquid paraffin. The skin showed some macroscopic reddening and thickening following the 5 per cent solution, and ulceration following the 10 per cent. Histologically it was noted that both solutions induced some epidermal necrosis, far more pronounced with the stronger solution; and in both cases there was

patchy epidermal hyperplasia. Four areas of skin on three rabbits were then treated with a single application of the carcinogen, and, after an interval of 1 month, applications twice weekly of croton oil (5 per cent) were begun. No tumors were recorded after 20 weeks.

The effects of arsenic and chloroform.—Arsenic trioxide and arsenic pentoxide were each made up as a 5 per cent preparation in a Lanette wax SX ointment base. The chloroform was applied undiluted. Four areas of skin of two rabbits were clipped free of hair, and to three of these was applied a single application of the carcinogen. After an interval of 1 month, the three areas were treated with one or another of the test compounds. The areas not treated with carcinogen were used as controls for the effects of the arsenic alone. The experiment was concluded at 20 weeks from the commencement of the second series of applications. No tumors were recorded.

GUINEA PIGS.

The effects of croton oil.—Studies on tumor formation with the guinea pig have been few, although Haagensen and Krebhiel (8) and Shimkin and Mider (16) have shown that the induction of sarcomas, using 3,4-benzpyrene and 20-methylcholanthrene, is possible within limits comparable to those occurring among other laboratory animals. Recently Berenblum (5) has shown that 9,10-dimethyl-1,2-benzanthracene is an effective carcinogen for guinea-pig skin, although the average latent period is as long as 51 weeks.

Preliminary skin tests on normal guinea pigs were carried out with 5 and 10 per cent solutions of croton oil in liquid paraffin. The 5 per cent solution induced considerable hyperplasia with minimal damage. Solutions were applied to both flanks of the animals, which were clipped free of hair with electric clippers. The effects of a limited number of applications of the carcinogen, followed by repeated applications of croton oil, were then investigated. Four groups of five guinea pigs were used for this:

Group 1.—both flanks were given three applications of carcinogen at twice-weekly intervals; after a 4-day interval only the left flanks received, twice weekly, 5 per cent croton oil applications.

Group 2.—the left flanks only were given three applications of the carcinogen; after a 4-day interval both flanks received twice-weekly applications of croton oil.

Group 3.—both flanks were given three applications of carcinogen; after a 4-day interval both received croton oil applications.

Group 4.—both flanks received but a single application of carcinogen, followed by the croton oil.

The experiment was continued for 33 weeks from the commencement of the croton oil applications, and no tumors were recorded. However, by the end of the experiment the animals were all in bad condition, having scratched the treated areas considerably. The procedure was then repeated on another group of 30 guinea pigs, divided into three groups of ten; only a single area of skin, in the interscapular region, was used for the applications in each animal. One group received ten applica-

TABLE 2

THE EFFECT OF IRRITANTS ON THE SKIN OF ANIMALS

Species	Agent tested	Skin histology*	Promotion test†
Rabbits	Wound healing‡		Several tumors induced
	20 per cent turpentine in liquid paraffin	Slight epidermal hyperplasia	No tumors induced
	5 per cent croton oil in liquid paraffin	"	"
	Chloroform	"	"
	5 per cent arsenic trioxide in Lanette wax SX	"	"
Rats	5 per cent croton oil in liquid paraffin	Some atrophic changes in dermis	"
	5 per cent croton oil in liquid paraffin	Marked epidermal hyperplasia	"

* On normal skin after 12 weekly applications of the agents.

† On skin painted with a single application of 9,10-dimethyl-1,2-benzanthracene (or more; see text), and then treated with the test agent twice weekly.

‡ Performed by punching a hole, 1 inch in diameter, through the ear with a sharp cork borer.

tions of carcinogen, and another five, at twice-weekly intervals, as it was considered that the three applications used originally might have been insufficient in this species. The third group received croton oil only. The experiment was continued for 36 weeks, but again no tumors were induced, and the skins of the animals were found to be in bad condition; a sensitivity to croton oil apparently developed after a few applications in some guinea pigs, which necessitated periodic cessation of treatment to prevent frank ulceration.

RATS.

The effects of croton oil.—Studies on tumor formation of the skin of rats are more numerous than those with guinea pigs, and recently Berenblum (5) has shown that 9,10-dimethyl-1,2-benzanthracene

3. Preliminary tests with croton oil induced macroscopic thickening and reddening; and an eczematoid lesion of the skin occurred with the 5 and 10 per cent solutions, more marked with the stronger. After four applications of croton oil given over 2 weeks some atrophic changes of the dermis were noted histologically, with minimal damage to the epidermis. In spite of this finding, twenty rats were each given three applications of the carcinogen at twice-weekly intervals, followed after 4 days by twice-weekly applications of 5 per cent croton oil. Another group of ten rats was given the croton oil only. This experiment was complicated by the fact that eczematoid skin lesions arose in all of the animals treated with croton oil. No tumors were recorded at 30 weeks from the commencement of the croton oil treatment.

DISCUSSION

Under the conditions of these experiments, croton oil possesses a definite specificity for the mouse, for which it is the most potent promoting agent yet investigated. The other substances tested for promoting activity in the mouse all proved negative following a single application of the carcinogen, although some of them had been shown earlier to have a co carcinogenic effect, in combination with repeated applications of carcinogenic hydrocarbons. It is possible that turpentine, for example, is a weak promoting agent, whose activity can only be revealed after more potent initiation.

In the rabbit, wound healing has proved to be by far the most effective method of promoting tumors, although it is likely that the turpentine sample used in this experiment differed in properties from that used by Rous and his collaborators. Inevitably, the continued use of such complex and uncontrolled organic mixtures will make the comparison of results haphazard. However, samples of croton oil, which are quite effective in the mouse, prove completely inactive in the rabbit. Chloroform, too, seems only a very weak promoting agent, requiring more than a single application of carcinogen preceding it, if its action is to be revealed (7).

The rat and the guinea pig are known to differ in their response to the carcinogenic hydrocarbons. In the previous analysis of the mechanism of carcinogenesis in mice (4) it was seen that the latent period was a function of the promoting action, whereas the total number of tumors induced was an expression of the initiating action only. Therefore, it might be concluded that, with an effective

promotion. Croton oil, however, appears to have no effect in either the rat or the guinea pig, although neither of the experiments could be described as final, in view of the poor health of the animals.

The results suggest that epidermal hyperplasia and tumor-promoting activity are not related in any simple fashion. Several of the substances investigated in the mouse, other than croton oil, induced marked hyperplasia, and yet were ineffective in promoting tumors following a single application of the carcinogen. It must be mentioned, however, that none of these substances seemed to induce as great a hyperplasia, and with such speed, as that encountered with croton oil, although this aspect requires further and more detailed histologic investigation. Nevertheless, this investigation raises the possibility of a specific metabolic role for croton oil in the mouse, rather than any simple explanation, as hitherto envisaged but not discussed. The only confusing factor is the undoubted effectiveness of wound healing as a promoting agent in the rabbit under these same conditions.

SUMMARY

1. Turpentine, acridine, fluorene, phenanthrene, castor oil, ricinoleic acid, glyceryl monoricinoleate, oleic acid, and silver nitrate have been tested for tumor-promoting activity following a single application of 9,10-dimethyl-1,2-benzanthracene in the mouse. They were ineffective as promoters of tumors.

2. The effects of wound healing, turpentine and croton oil applications, following a single application of 9,10-dimethyl-1,2-benzanthracene, have been tested for tumor-promoting activity on the rabbit. Wound healing alone has been found to be an effective stimulus.

3. The effects of repeated applications of croton oil, following a minimal number of applications of 9,10-dimethyl-1,2-benzanthracene have been investigated in the guinea pig. No tumors were induced.

4. The effects of croton oil, following three applications of 9,10-dimethyl-1,2-benzanthracene in the rat, have been investigated. No tumors were induced.

5. The specific effect of croton oil on the mouse and of wound healing on the rabbit, as promoters of tumor formation are discussed.

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The Possible Carcinogenicity of Overcooked Meats, Heated Cholesterol, Acrolein, and Heated Sesame Oil*

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INTRODUCTION

This paper is concerned with studies of the carcinogenicity of several heated substances, in a preliminary survey of certain dietary constituents as possible extrinsic, environmental causes of cancer.

The cause of the high incidence of cancer of the alimentary tract, particularly of the stomach and colon, in Europeans and Americans remains unexplained. There is also no adequate explanation for the comparatively low incidence of these cancers in certain primitive peoples, notably the Bantu of the East Coast and of South Africa (5, 6, 27, 37), and the Javanese (8, 9), whereas both these races are subject to much primary liver cancer. Even more striking is the observation that in contrast to the low incidence of primary cancer of the stomach and large bowel and the high incidence of hepatic cancer in the African Bantu, the incidence at these two sites is reversed in the Afro-American, whose distribution of these cancers comes to resemble that of whites in America (21, 17). While negroes in America are not all Bantus, surely some of them are (21). The comparatively sudden change in incidence of these different types of primary cancer suggests that environmental rather than hereditary factors may be playing the dominant role in their causation.

This assumption is supported by another line of evidence; namely, that obtained from the examination of negro livers for a carcinogen. Thus the noncancerous livers of the African Bantu commonly contain a carcinogen according to des Lignier (14), those of the Afro-American only rarely (34).

If cancers of the human alimentary tract are induced by exogenous carcinogens derived from the environment, the portal of entry would probably be the mouth, and the carcinogenic factor would almost of necessity be carried by food or drink. It is known from the work of Lorenz and Stewart that carcinogenic hydrocarbons taken by mouth can cause cancer

of the stomach (22) and of the intestine (23) in mice. It is also known that the diet of the South African Bantu is different from that of the Afro-American, and that the Javanese eat food different from that of other races living in Java which have an entirely different tumor incidence (8).

From what is known about the chemistry of the synthetic carcinogens (13, 16) one of the most probable dietary sources of carcinogens would be meats or meat products, or vegetable oils, which had been subjected to high temperatures in cooking. Indeed, there are numerous reports which state that such products have carcinogenic activity for animals.

In testing food products for carcinogenicity rats and mice have been used almost exclusively. In evaluating the outcome of experiments it is important to know that these species react differently. Rats on the whole have been much more susceptible than mice to tumor induction by injection and by feeding. Little is known about the reaction of rats to skin painting by these foodstuffs, while mice, according to the few experiments in which they were painted, appear to have no unusual degree of susceptibility.

A great amount of work has been done, since the report by Singer in 1913 (32), on the production of gastric lesions in rats by various diets that are deficient in total caloric value, in certain of the principal food constituents, or in vitamins. In general, the lesions develop quickly, and consist of ulcers or papillomas with the occasional production of infiltrative lesions later. Since this paper is not primarily concerned with unbalanced diets, reference is made to recent papers and reviews by Morris and Lippincott (25), Beck and Peacock (4), Brunschwig and Rasmussen (11), and Klein and Palmer (20), where details may be found.

Of greater immediate interest are the various animal and vegetable substances that have been tested for carcinogenicity either because of direct interest in the problem as related to foodstuffs, or because they were used as solvents in other experiments. Kennaway and Sampson in 1928 (18) painted 70 mice twice weekly with the tar obtained by heating cholesterol to 800-

* This investigation was aided by grants from the National Advisory Cancer Council and the Commonwealth Fund.

810° C. The first tumor appeared on the 83rd day. The total number was 18, of which 11 were considered malignant. Three years earlier Kennaway (19) had made cancer-producing tars from human skin and from yeast by heating to 920° C.

Barry and Cook (2) stated that injection of lard elicited granulomas, but not tumors, in 2 out of 10 rats, and that it produced no tumors in 20 mice, of which the longest survival was 581 days. Andervont (1) injected mice with a total of 1 cc. of lard, given in 3 injections in 3 months, and induced no tumors in 190 mice in 189 days. Burrows, Hieger, and Kennaway (12) summarized numerous experiments involving the injection of various oils and fats. In 236 mice no tumors arose, but in rats the injection of lard was followed by 7 sarcomas in 134 animals, and 1 sarcoma was induced by olive oil plus arsenious acid. Lard heated to 340–360° C. was also not carcinogenic for 12 mice but induced 2 sarcomas in 14 rats.

Schabad (31) injected sunflower oil into 16 mice in quantities of 0.25 to 0.50 cc. on 3 occasions, but no tumors had appeared after 6¼ months. Recently, Beck (3) heated cottonseed oil to 340–360° C. for 1 hour in air, or repeatedly at 200–210° C. for a total of 12 hours. The first sample induced 2 sarcomas on subcutaneous injection in 0.5 cc. doses, at 414 and at 535 days, in 6 mice, while the latter elicited none. Rowntree and his coworkers (30) induced abdominal sarcomas by feeding rats a crude wheat germ oil. This work has not been confirmed by others (10).

Widmark (40) painted mice with alcohol or petroleum ether extracts of horse muscle heated to 275° C., browned butter, or roasted coffee, but no sarcomas were induced. Of 241 mice, 23 females survived for 11 months. Nine of them developed mammary gland tumors, an incidence of 40 per cent compared with a normal incidence of 10 per cent for this stock. Two of these tumors followed the petroleum ether extract of roasted coffee, 2 the similar extract of roasted muscle, 1 this extract of browned butter, and 4 the alcohol extract of roasted muscle. He also induced 1 sarcoma in a mouse painted with cholesterol treated at 275° C.

The strongest claims for the carcinogenic activity of heated foodstuffs have come from the laboratories of R. G. Coffo and of Waterman. In a series of papers R. G. Coffo (28, 29) described the induction of benign and malignant tumors in the stomach and liver in rats by feeding overheated cholesterol, and animal and vegetable fats. He used lard, veal fat, mutton fat, and olive oil, heated to 350° C. for 30 minutes. He thought that the cholesterol was oxidized to a carcinogenic chemical. Waterman (39) fed cholesterol oleate to mice and obtained malignant tumors in the stomach after about 1 year. Veldstra (38) thought that the

active substance in cholesterol oleate was Δ^5 -cholesta-2,6-diene. Domagk (15) described a tumor of the stomach in a mouse after prolonged feeding of a diet rich in olive oil. A critical analysis of the chemical work in these papers has been made by Cook and Kennaway (13), and of the pathology by Klein and Palmer (20).

EXPERIMENTAL

In presenting the experimental results tables are given to show the sex and the number of mice used in testing the various substances for carcinogenicity, the number surviving at various periods of time, and the number of tumors induced. These data are not repeated in the text, where only the method of preparation of the extracts, the details of dosage, the general reactions, and details respecting the tumors are given. All injections were made subcutaneously in the interscapular region.

It may be stated here that four sarcomas were induced in these experiments. Of these, one is described in experiment 4, and three in experiment 17.

The mice used in experiments 1 to 6 and 13 to 17 inclusive were from our own stock of partly inbred albinos. Their susceptibility to tumor induction and something about their incidence of spontaneous tumors have been described in several publications (33, 35, 36). They are susceptible to subcutaneous sarcoma induction but sarcomas do not arise in them spontaneously. In experiment 7 the mice used were first generation offspring of C57 brown breeding stock obtained from the Roscoe B. Jackson Memorial Laboratory. All others used in these experiments were purchased from dealers, and nothing is known about their susceptibility to tumors. The mice were usually about 3 months of age when the experiments were begun, although a few were younger and others older.

CARCINOGENICITY OF MEAT EXTRACTS

Preparation of meat extracts used in experiments 1 to 7.—Twenty pounds of good grade beef, 20 pounds of good pork, and 5 pounds of suet were ground up together, mixed thoroughly, formed into small meat balls, and fried in an open pan. The frying was carried out to a crisp brown, somewhat past the point generally used in the preparation of human food, but without burning. The fat which drained away readily in the melted state was poured off. It weighed 3,280 gm. and was used without further change for injection in experiment 1 and for feeding in experiment 2.

A 300 gm. portion of the crude fat thus obtained was saponified with 1.5 liters of alcohol and 300 gm. of sodium hydroxide in 300 cc. of water for 1 week at room temperature. The mixture was then diluted with water and extracted 3 times with benzene. The ben-

zene extracts were pooled and washed 3 times with water, the emulsion being broken by alternate freezing and thawing. The benzene extract was finally evaporated to dryness, yielding 1.14 gm. of an oil. This was made up in sesame oil so that 0.5 cc. contained 0.1 gm. of extract. This was used for injection in experiment 3.

The fried meat was reground and extracted with 1 volume of acetone for 24 hours. This procedure was repeated twice. The meat residue was allowed to dry. The acetone extracts were combined and evaporated to a small volume, when an aqueous and a fatty phase separated. The aqueous phase was diluted again with acetone and reevaporated until no more acetone came off. A new fatty phase sepa-

dissolved in sesame oil so that 0.5 cc. contained 0.15 gm. This was used for injection in experiment 7.

Experiment 1. Injection of fat from overfried mixed meats.—Twenty mice were injected each with 0.5 cc. of the warmed fat which had been poured from overfried meats. At intervals of 4 weeks the injections were repeated so that each mouse had a total of 5 injections within a period of 4 months. The total dose for each mouse was 2.5 cc.

This fat was well tolerated. It persisted in the form of soft masses for several months, but no mouse autopsied after 11 months showed any unabsorbed residue. See Table 1 for results.

Experiment 2. Feeding of fat from overfried meats.—Thirty mice were placed on the stock diet to

TABLE 1: CARCINOGENICITY OF MEAT EXTRACTS FOR MICE

Experiment No.	Male mice	Female mice	Total mice	Survival time in months										Sarcomas induced
				6	9	12	15	18	21	24	27	30	33	
1		20	20	20	20	19	18	15	13	5	3	1	0	0
2		30	30	30	29	27	15	11	4	4	1	0	0	0
3		10	10	10	10	8	8	7	5	2	1	0	0	0
4		20	20	20	20	18	16	10	7	3	2	1	0	1
5		30	30	29	26	15*	13	10	6	3	0			0
6		8	8	8	8	7	6	6	4	0				0
7	9		9	9	9	9	5	0						0
8 A	24	24	48	18	13	9	0							0
8 B		10	10	1	1	0								0
9	58	24	82	21	7	1	0							0
10	36	12	48	9	2	1	0							0
11		26	26	10	0									0
12 A		25	25	7	0									0
12 B		21	21	6	1	1	0							0

* Ten mice killed for gastric examination.

rated. The fatty phases were combined. This fat weighed 403 gm.

The dried meat residue (about 5,500 gm.) was extracted with benzene in continuous extractors. The tissue was ground in a Wiley mill and reextracted with benzene. The benzene extracts were combined, filtered, and evaporated to dryness. This fat weighed 1,770 gm. It was used for injection in experiment 4 and for feeding in experiment 5.

A 300 gm. portion of this benzene extract fat was saponified by the methods employed for the supernatant fat mentioned above. The yield of nonsaponifiable material was 0.94 gm. of an oil. It was made up in sesame oil so that 0.5 cc. contained 0.1 gm. of extract, and used for subcutaneous injection in experiment 6.

The fat from the acetone extraction (403 gm.) mentioned above was dissolved in benzene and extracted with 50 per cent alcohol. The benzene was evaporated to dryness, leaving 330 gm. of oily residue. Of this 300 gm. was saponified by the methods given above. The 2.0 gm. of nonsaponifiable material obtained was

which 5 per cent by weight of this meat fat was added. For a period of about 3 weeks early in the course of the experiment the amount of fat added to the diet was 10 per cent. The mice thrived on this diet. Each consumed about 145 mgm. of fat daily for 14 months.

Experiment 3. Injection of the nonsaponifiable lipids from overfried mixed meats.—Ten mice were injected each with 100 mgm. of the nonsaponifiable lipids from the fat used in experiment 1, dissolved in 0.5 cc. of sesame oil. This material was well retained, and many mice showed traces of it when autopsied more than a year later. Each mouse received the nonsaponifiable lipids from 30 gm. of the original fat.

Experiment 4. Injection of a benzene extract from overfried mixed meats.—The fat obtained by benzene extraction was injected into 20 mice. Each was given 0.5 cc., followed at intervals of 4 weeks by injections of the same size. Each mouse had a total of 5 injections (2.5 cc. of fat) within a period of 4 months. This fat, which was well tolerated by the mice, per-

sisted longer than did the fat in experiment 1. At autopsy masses of it were seen as long as 18 months after the first injection.

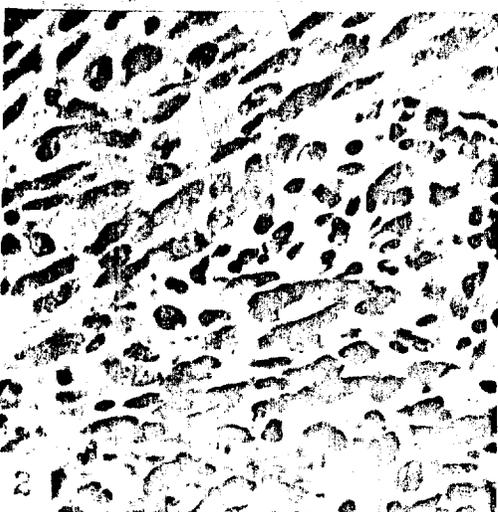
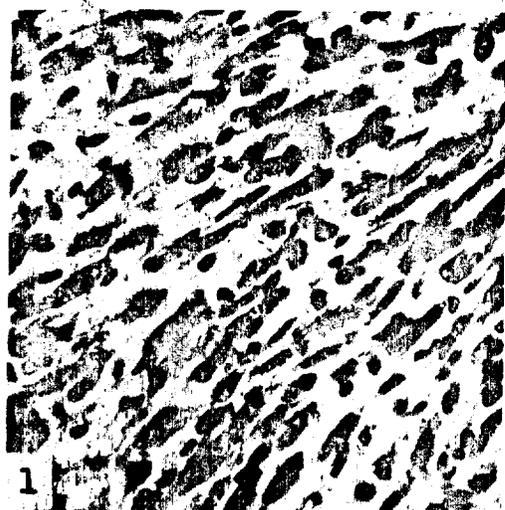


Fig. 1. Spindle cell sarcoma induced in experiment 4 at the site of injection of a benzene extract from overfried mixed meats. Mag. $\times 420$.

Fig. 2. Spindle cell sarcoma induced by heated sesame oil 18 months after the first injection. Mag. $\times 420$.

The mouse developed a subcutaneous sarcoma in the left groin with a mass of the fat and died 424 days after the first injection. Besides a subcutaneous tumor measuring 20×15 mm. in the right lateral chest region which had the gross and microscopic appearance of a primary gland carcinoma, and 2 lung nodules each 2 mm. in diameter which microscopically proved to be primary lung tumors, this mouse had an oval sub-

cutaneous mass $22 \times 12 \times 10$ mm. located in the left groin. About one-third of this mass consisted of hardened white fat and the other two-thirds appeared like homogeneous pinkish-white tumor, which partly enclosed the fat. Upon microscopic examination it was found to be a spindle cell sarcoma. (See Fig. 1.)

Experiment 5. Feeding of a benzene extract from overfried mixed meats.—Thirty mice were placed on the stock diet to which 5 per cent by weight of this fat was added. For a period early in the experiment the amount of fat added was 10 per cent. The animals appeared to thrive on this mixture and it was fed until the supply was exhausted, at 330 days. Each mouse consumed about 145 mgm. of fat per day.

When the feeding of fat was discontinued 10 mice were sacrificed and their stomachs examined. They showed no recognizable lesions, nor did any of the others subsequently develop tumors of the alimentary tract.

Experiment 6. Injection of nonsaponifiable lipids of a benzene extract from overfried mixed meats.—Eight mice were injected each with 100 mgm. of this extract dissolved in 0.5 cc. sesame oil. Although it was retained no sarcomas developed. The 100 mgm. which each mouse received represented the nonsaponifiable lipids from 37.5 gm. of the original fat which had been used in experiments 4 and 5.

Experiment 7. Injection of the nonsaponifiable lipids from the benzene-soluble material of an acetone extract of overfried mixed meats.—Nine mice were each given 1 injection consisting of 150 mgm. of this extract in 0.5 cc. of sesame oil. This represents about 22.5 gm. of the original fat, which was fairly rich in nonsaponifiable material.

PREPARATION AND TESTING OF MEAT EXTRACTS USED IN EXPERIMENTS 8 TO 10

Ten pounds of fresh boiling beef, 10 pounds of fresh pork shoulder which was rather fatty, and 1 pound of suet were ground up together. Half of this was saponified with 450 gm. of potassium hydroxide and 2 liters of 95 per cent alcohol on a steam bath and the remainder divided into 2 equal portions, of which one was overfried and the other overbaked.

The meat to be fried was moulded into flat cakes and heated in an open pan until the surfaces were brown and crisp, although the inside was still soft. Saponification with potassium hydroxide was then carried out on a steam bath for 3 days, water being added.

The meat used for baking was moulded into a loaf and baked until the surface was brown and crisp, a point somewhat beyond that usually used for food. The temperature in the oven was between 185° and 220° C. for a period of $3\frac{1}{2}$ hours. This meat was then also saponified.

After saponification the three meat portions—raw, fried, and baked—were separately extracted 5 times with ethylene dichloride, the extract was washed with water, dehydrated with anhydrous sodium sulfate, filtered, and distilled to small volume in partial vacuum. The oily residues were resaponified with alcoholic potassium hydroxide, and the final nonsaponifiable extracts obtained were: Raw meat, 3.1 gm.; fried meat, 3.1 gm.; baked meat, 2.8 gm. These extracts were made up in sesame oil so that 0.1 cc. contained 25 mgm. of the extract and tested for carcinogenic activity by subcutaneous injection into mice (experiments 8, 9, and 10).

Experiment 8 A. Injection of raw mixed meat extract.—Forty-eight mice were given 1 injection con-

ethylene dichloride, and the combined extracts were dried with sodium sulfate and evaporated *in vacuo*. The 3.0 gm. of residue was dissolved in sesame oil. Twenty-six mice were given 1 injection of 30 mgm. of this extract in 0.1 cc. of sesame oil.

Experiment 12. Nonsaponifiable lipid extract of mixed meats.—A mixture of equal parts of beef, pork and lamb with a total weight of 10 pounds was treated exactly as was the beef in experiment 11. The final nonsaponifiable extract weighed 3.6 gm. In experiment 12 A 26 mice were each given 1 injection of about 36 mgm. of this extract dissolved in 0.1 cc. of sesame oil. When this induced no tumors the same extract was used in doses 3 times as large; namely, 109 mgm. per mouse (experiment 12 B).

TABLE II: CARCINOGENICITY OF MISCELLANEOUS SUBSTANCES

Experiment No.	Substance tested	Male mice	Female mice	Total mice	Survival time in months						Sarcomas induced	
					6	9	12	15	18	21		24
13	Unheated cholesterol	18		18	12	9	7	2	0			0
14	Cholesterol, 200° C.	20		20	18	8	2	1	0			0
15	Cholesterol, 300° C.	14	16	30	16	10	6	3	2	0		0
16	Acrolein		15	15	14	14	11	6	3	1	0	0
17	Heated sesame oil	16	15	31	26	15	9	6	4	1	0	3

sisting of 25 mgm. of the nonsaponifiable lipids in 0.1 cc. of sesame oil. When this induced no tumors a larger amount was tried (Experiment 8 B).

Experiment 8 B. Injection of raw mixed meat extract.—In this experiment 100 mgm. of nonsaponifiable lipids used in experiment 8 A was dissolved in 0.2 cc. of sesame oil and injected into each of 10 mice.

Experiment 9. Injection of fried mixed meat extract.—Eighty-two mice were injected with 25 mgm. of this nonsaponifiable lipid extract in 0.1 cc. of sesame oil.

Experiment 10. Injection of baked mixed meat extract.—Forty-eight mice were injected with 25 mgm. of this extract dissolved in 0.1 cc. of sesame oil.

Experiment 11. Nonsaponifiable lipid extract of beef.—This extract and that used in experiment 12 were prepared by Dr. Carl Marberg, who kindly gave his permission to include this work. Ten pounds of fresh beef of average fatness was ground and made into meat balls. These were fried until they were well done—hard and brown, but not burned. The meat, together with the fat, was transferred to a flask and treated with an alcoholic solution of potassium hydroxide. After 2 hours a volume of water equal to that of the alcohol was added and the material was refluxed on the steam bath overnight. The next day the nonsaponifiable material was extracted 5 times with ethylene dichloride. The ethylene dichloride was evaporated *in vacuo*, and the residue was resaponified with alcoholic potassium hydroxide. After dilution with water the soap solution was again extracted with

PREPARATION AND TESTING OF THE CHOLESTEROL USED IN EXPERIMENTS 13, 14, AND 15

The cholesterol for experiment 13 was freed from 7-dehydrocholesterol and impurities causing general absorption in the ultraviolet range by treatment with bromine according to the method of Bills, Honeywell, and MacNair (7). Ten grams of the purified cholesterol was incorporated in 100 cc. sesame oil. For experiment 14, 10 gm. of the purified cholesterol was heated in a 25 × 200 mm. open tube at 200° C. for 2 hours by immersion in an oil bath. After cooling the mass was dissolved in 95 per cent ethanol, transferred to 100 cc. sesame oil, and the alcohol removed by evaporation under diminished pressure. For experiment 15 the same procedure was followed, but at 300° C.

Experiment 13. Unheated cholesterol.—Eighteen mice were injected each with 50 mgm. of cholesterol suspended in 0.5 cc. of sesame oil. At intervals of 4 weeks the injections were repeated, so that a total of 200 mgm. of cholesterol was administered to each mouse in a period of just under 3 months. This material was well tolerated. Some mice showed persistent masses at the injection site.

Experiment 14. Cholesterol heated to 200° C.—Twenty mice were injected each with 50 mgm. of this cholesterol on each of four occasions, the conditions being exactly as in experiment 13. This material was well tolerated.

Experiment 15. Cholesterol heated to 300° C.—

Thirty mice were injected each with 200 mgm. of this cholesterol divided into 4 doses exactly as in experiment 13.

CARCINOGENICITY OF ACROLEIN

Experiment 16.—Acrolein was made up so that 10 mgm. was contained in 0.5 cc. of sesame oil. When 10 mice were injected each with this amount they quickly became excited, went into shock, and died within 1 hour. When the amount of acrolein contained in 0.5 cc. of sesame oil was reduced to 1 mgm. and this was injected into each of 2 mice they both died within 7 hours. Consequently the dose adopted was 0.2 mgm. of acrolein dissolved in 0.1 cc. of sesame oil. This amount was injected into each of 15 mice at weekly intervals for 24 weeks. The total amount for each mouse was 4.8 mgm. of acrolein and 2.4 cc. of sesame oil.

CARCINOGENICITY OF HEATED SESAME OIL

Experiment 17.—Because of its wide use as a vehicle for many biological substances sesame oil was selected as an example of a vegetable oil for heating. It was slowly heated to 350° C. in an open vessel, and then allowed to cool. It began to darken and to fume vigorously at about 320° C.

This oil was injected into 31 mice. Each mouse was given 0.5 cc., followed at intervals of 4 weeks by 2 additional injections. Thus each mouse had a total of 1.5 cc. The material was well tolerated.

Three sarcomas occurred at the site of injection. Two were in males and one in a female. The first was discovered in the 12th month and the animal died in the 13th month with a tumor 35 × 30 × 18 mm. which had ulcerated through the skin and infiltrated the muscles beneath. It was in direct contact with a mass of oil. Microscopic examination showed it to be a spindle cell sarcoma. There were no metastases.

The second sarcoma, discovered in the 20th month, killed its bearer before the month was out. It was 20 mm. in greatest diameter and had infiltrated both the overlying skin and the underlying muscle. Residual sesame oil was in contact with it. It, also, proved to be a spindle cell sarcoma; there were no metastases. (See Fig. 2.)

The third sarcoma was found in a female mouse, the last survivor in the experiment, that died on the 10th day after the first injection. The tumor, 6 × 15 × 5 mm., was in direct contact with masses of injected oil. Microscopic examination showed this to be a spindle and mixed cell sarcoma.

MISCELLANEOUS TUMORS

In some of these experiments growths were encountered also in the mammary gland, the lungs, and

the lymphatic system. The C57 brown mice had no such tumors, and the mice purchased on the open market had only a few, while the mice of our own stock had many more but well within the incidence previously reported (35). As there was no evidence that the incidence of these tumors was increased by the various substances fed or injected, the growths are considered spontaneous.

COMMENT

Four sarcomas were induced at the sites of injection of these various substances in experiments in which the total number of mice was 522, the number of 6 month survivors was 284, and the number surviving for over 12 months was 150, as is shown in the tables. In experiments 8 to 12 the mortality was unfortunately very high early in the course of the experiments. Taken alone, these experiments are not very significant, even though a few animals survived for periods of time which some have considered adequate. The results are included, however, because so far as they go they substantiate the results of the other experiments with meat extracts.

Though the experiments reported here are not exact duplicates of work reported by anyone else, they permit certain comparisons and correlations with respect to the sesame oil, the meats, and the cholesterol.

Sesame oil.—At 12 months the number of mice living that had been injected with unheated sesame oil, used as a vehicle for other substances in these experiments, was 61. None developed tumors at the site of injection. At the same time 9 mice were living that had been injected with sesame oil heated to 350° C., and of these, 3 developed sarcomas. Carcinogenic activity was therefore probably acquired in the heating of this oil. In other experiments (35, 33) unheated sesame oil likewise did not induce tumors in these mice. The heated sesame oil persisted longer at the site of injection than did the unheated oil, a factor that may be significant.

Schabad (31) reported that sunflower oil was not carcinogenic for mice, and Burrows, Hieger, and Kenaway (12) found this to be true for olive oil, linseed oil, and wheat germ oil. Beck (3), on the other hand, found cottonseed oil carcinogenic after heating to 340–360° C. for 1 hour.

When the evidence that heated vegetable oils are carcinogenic for rats is added to that obtained for mice it becomes evident that an exhaustive study of the carcinogenicity of various heated vegetable oils is imperative.

Meats.—The case for the carcinogenicity of the animal fats is not so convincing. One mouse injected with fat extracted with benzene from a mixture of overheated beef and pork died with a sarcoma at the

point of injection after 424 days. This tumor occurred in 1 of 18 mice that lived for 12 months or longer. However, at this time an additional 55 mice which had received injections of similar fats, or the nonsaponifiable fractions thereof, were living, as were 42 that had been fed these fats, yet none developed sarcomas. It is possible, of course, that the active substance is not present in the nonsaponifiable fraction, and that this was not the best method of concentrating the active principle.

Barry and Cook (2), Andervont (1), Burrows, Hieger, and Kennaway (12), and others, have injected animal fat into mice with negative results, and the group last named also injected lard which had been heated at 340-360° C. without inducing tumors, although the number of mice used was small.

TABLE III: COMMERCIAL MANUFACTURE OF LARD IN THE UNITED STATES*

Method	Prepared by this method, per cent	Maximum temperature reached, ° C.
Steam-rendered	80	150
Open-kettle-rendered	5	120
Dry-rendered	15	130

* Data supplied by Dr. Frank C. Vibranis, Chief Research Chemist, American Meat Institute, Chicago.

Even though injection experiments in mice have been uniformly negative in the hands of others, it is possible that the one tumor reported here may have been induced. The temperature was probably higher than that used in the commercial preparation of lard, which is said not to exceed 150° C. (26), but natural conditions in preparing meats for food were simulated, although slightly exaggerated.

The maximum temperatures used in the preparation of meats for human consumption are usually about 150° C., and are said rarely to exceed 200° C. This temperature, also, is seldom exceeded in the use of animal fats for deep frying (24), as in the preparation of doughnuts and potato chips, but here the fats are heated at this temperature repeatedly and for long periods of time. It is possible that harmful chemical reactions might occur gradually under these conditions which, at higher temperatures, might take place quickly.

Under these circumstances this subject merits further study despite the small amount of positive evidence presented here.

Cholesterol.—The situation with respect to cholesterol is less clear. The experiments of Kennaway and Sampson (18), who heated cholesterol to 800-810° C., thus forming a tar which was carcinogenic, are not comparable with those reported here. Neither are the experiments of Roffo (28, 29) because he fed rats, and their stomachs appear to be highly sensitive to

many foodstuffs. The observation of Widmark (40), who induced one sarcoma in a mouse painted with cholesterol treated at 275° C., is difficult to evaluate in the absence of further information. It appears, then, that a final decision should be postponed until more work has been done with cholesterol.

SUMMARY

Numerous extracts made from overfried and baked meats were tested for carcinogenicity in mice by subcutaneous injection and by feeding.

1. The fats from a mixture of beef, pork, and suet that had been overfried were tested by injection, by feeding, and by injection of the nonsaponifiable lipids.

2. The nonsaponifiable lipids from a second mixture of beef, pork, and suet prepared from the raw meat, after overfrying and after-overbaking, were tested by injection.

3. The nonsaponifiable lipids from overfried beef were tested by injection.

4. The nonsaponifiable lipids from a mixture of beef, pork, and lamb were tested by injection.

Only one sarcoma was induced at the site of injection of these meat extracts. This occurred in a mouse injected with the benzene-soluble fats from overfried mixed meats.

Pure cholesterol heated to 200° C. and to 300° C. was not carcinogenic on subcutaneous injection into mice. Aerolein also was not carcinogenic. Sesame oil that had been heated to 350° C. was carcinogenic, inducing 3 sarcomas in 9 mice that lived for more than one year.

These results, taken with those of others, indicate that further study of the carcinogenic potency of heated vegetable fats appears to be warranted.

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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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* 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 ₃ (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 H ₂ O	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 B₁₂ (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

† 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*

Additions to basal A	4 wk. wts. (gms.)	Experiment 1	
		Gm. feed 4 wks.	Time of feed passage (minutes)
None	292	2.02	146
None	288	2.17	132
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
2.5% Prime tallow	300	1.96	144
5% No. 1 tallow	322	1.93	
5% Commercially stabil- ized fat*	293	1.94	
5% Hydrogenated fat†	289	2.03	

* Sta-fat—Darling Manufacturing Co., Chicago, Illinois.

† Hydropoid—Bowman Feed Products, Inc., Holland, Michigan.

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.

The feces samples were collected on the second and fourth 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 commercially 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	% fat in dry feces	% FFA of feces fat
		Gm. wt.		
None	283	2.14	1.18	35
None	325	2.09		
5% white grease	311	1.92	3.17	64
10% white grease	289	1.91	5.80	59
5% Oleic acid	292	1.96	3.68	67
5% Hydrogenated fat†	309	2.21	9.27	82

† 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 lingo-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	1.76

* 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 doubtful 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

	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

	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% linolenic acid	281	1.99
Basal + 5% linoleic 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

	Wt. (gms.)	Gms. feed	% fat in dry feces
		Gm. wt.	
Basal diet A	302.2	2.04	1.12
Basal + 5% crude tall oil	312.5	2.05	1.20
Basal + 5% tall oil	155.8	3.05	—
Basal + 5% rosin acids	201.7	3.01	2.44
Basal + 5% oleic acid	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 ¹ /A	B ¹ /B
	A	B	A ¹	B ¹		
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 Activity¹

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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 reactive substances.

Oxidation products from fats and fatty foods, as well as those from the atmosphere, are adventitious sources of oxidizing products. Inhalation of tobacco smoke represents a nonadventitious and deliberate regimen for introducing oxidizing 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 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 I
Compounds evaluated

No.	Compound	Structure	Molecular weight
<i>Fatty acids and derivatives</i>			
I	Lauric acid	$\text{CH}_3(\text{CH}_2)_{10}\text{CO}_2\text{H}$	200.3
II	Palmitic acid	$\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H}$	256.4
III	Oleic acid	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	282.5
IV	Stearic acid	$\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H}$	284.5
V	4-Ketostearic acid	$\text{CH}_3(\text{CH}_2)_{13}-\overset{\text{O}}{\underset{\text{ }}{\text{C}}}-\text{CH}_2\text{CO}_2\text{H}$	298.5
VI	2-Hydroxystearic acid	$\text{CH}_3(\text{CH}_2)_{15}-\overset{\text{OH}}{\underset{ }{\text{C}}}-\text{CO}_2\text{H}$	300.5
VII	12-Hydroxystearic acid	$\text{CH}_3(\text{CH}_2)_5-\overset{\text{OH}}{\underset{ }{\text{C}}}-\text{CH}-(\text{CH}_2)_{10}-\text{CO}_2\text{H}$	300.5
VIII	Methyl stearate	$\text{CH}_3-(\text{CH}_2)_{16}-\text{CO}_2\text{CH}_3$	298.5
IX	Methyl 12-hydroxystearate	$\text{CH}_3(\text{CH}_2)_5-\overset{\text{OH}}{\underset{ }{\text{C}}}-\text{CH}(\text{CH}_2)_{10}-\text{CO}_2\text{CH}_3$	314.5
X	9(10)-Monohydroxystearic acid	$\text{CH}_3-(\text{CH}_2)-\overset{7(8)}{\underset{\text{OH}}{\text{C}}}-\text{CH}-(\text{CH}_2)-\overset{8(7)}{\text{C}}-\text{CO}_2\text{H}$	300.5
XI	Stearohydroxamic acid	$\text{CH}_3(\text{CH}_2)_{16}\text{CONHOH}$	299.5
<i>Lactones</i>			
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
<i>Epoxy compounds</i>			
XXII	<i>cis</i> -9,10-Epoxy stearic acid	$\text{CH}_3(\text{CH}_2)_7 \begin{array}{c} \text{H} \quad \text{H} \\ \diagdown \quad / \\ \text{C} \quad \text{C} \\ / \quad \diagdown \\ \text{O} \end{array} (\text{CH}_2)_7\text{CO}_2\text{H}$	298.5
XXIII	<i>trans</i> -9,10-Epoxy stearic acid	$\text{CH}_3(\text{CH}_2)_7 \begin{array}{c} \text{H} \quad \text{H} \\ \diagdown \quad \diagdown \\ \text{C} \quad \text{C} \\ / \quad / \\ \text{O} \end{array} (\text{CH}_2)_7\text{CO}_2\text{H}$	298.5
XXIV	Glycidyl laurate	$\text{CH}_3(\text{CH}_2)_{10}\text{CO}_2\text{CH}_2\text{CH} \begin{array}{c} \diagdown \\ \text{O} \\ \diagup \end{array} \text{CH}_2$	296.4
XXV	Glycidyl oleate	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{CH}_2\text{CH} \begin{array}{c} \diagdown \\ \text{O} \\ \diagup \end{array} \text{CH}_2$	352.6
XXVI	Glycidyl stearate	$\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{CH}_2\text{CH} \begin{array}{c} \diagdown \\ \text{O} \\ \diagup \end{array} \text{CH}_2$	348.6
XXVII	<i>p</i> -Nitroperoxybenzoic acid	$\text{O}_2\text{N} \begin{array}{c} \diagdown \quad \diagup \\ \text{O} \\ \diagup \quad \diagdown \end{array} \text{CO}_2\text{H}$	183.4
XXVIII	Peroxy lauric acid	$\text{CH}_3(\text{CH}_2)_{10}\text{CO}_3\text{H}$	216.3
XXIX	Lauroyl peroxide	$\text{CH}_3(\text{CH}_2)_{10} \begin{array}{c} \text{C} \quad \text{O} \quad \text{O} \quad \text{C} \\ \diagdown \quad \diagup \quad \diagdown \quad \diagup \\ \text{O} \quad \quad \quad \text{O} \end{array} (\text{CH}_2)_{10}\text{CH}_3$	378.6

Chemicals, Rochester, N. Y., was the source of β -butyrolactone (XII), γ -butyrolactone (XIII), and 2,2,4-trimethyl-3-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-Epoxy stearic acids were prepared by epoxidation 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). Peroxy lauric 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 tricaprilyn 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 strains. 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						Tumors by month of appearance		
							0	6	9	12	15	18	Subcutaneous sarcomas	Pulmonary tumor	Other tumors
<i>Controls</i>															
	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	51	21	14	14, 17, 17, 17, 17	B(11, 14, 15, 17, 17, 17, 17, 17, 18, 18), C(14, 15)
	None	1965				C	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	
<i>Fatty acids and esters</i>															
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	6	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	57.0/11.4	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-Hydroxy-stearic acid	1965	1.25	2/10.4	130/10.4	C	15	14	14	14	14	11		23, 23	
VII	12-Hydroxy-stearic acid	1965	1.0	2/80	80/8	S	15	14	10	8	8	6		14	L(11)
	12-Hydroxy-stearic 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-Hydroxy-stearic 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 triacrylin)	Mouse strain	Mice alive at month						Tumors by month of appearance		
							0	6	9	12	15	18	Subcutaneous sarcomas	Pulmonary tumor	Other tumors
<i>Lactones</i>															
XII	<i>n</i> -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	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			
	γ -Stearolactone	1965	0.05	2/80	4/8	S	15	13	7	4	0		4, 6, 6, 8, 9, 9, 9, 9, 12	15	
	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	
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	
	Stearolactone	1967	0.005	1/26	1.3/2.6	S	16	16	15	15	12		18	15, 18	
<i>Epoxy compounds</i>															
XXII	<i>cis</i> -9,10-Epoxy-stearic acid	1965	1.0	2/114	114/11.4	C	10	9	9	9	7	4	18	20	C(13)
	<i>cis</i> -9,10-Epoxy-stearic acid	1965	0.5	2/114	57/11.4	C	10	10	10	10	8	7	11		
	<i>cis</i> -9,10-Epoxy-stearic acid	1965	0.005	2/80	4/8	C	10	9	8	7	7	7	6	21	K(21)
	<i>cis</i> -9,10-Epoxy-stearic acid	1965	1.0	2/82	82/8.2	S	15	14	13	11	8	5			L(14)
	<i>cis</i> -9,10-Epoxy-stearic acid	1965	0.5	2/82	41/8.2	S	15	13	10	9	7	5	10, 18	17	G(17)
	<i>cis</i> -9,10-Epoxy-stearic 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	<i>trans</i> -9,10-Epoxy-stearic 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	
<i>Peroxy compounds</i>															
XXVII	<i>p</i> -Nitroperoxy-benzoic acid	1964	1.0	2/114	114/11.4	C	10	8	7	4	4	3			
	<i>p</i> -Nitroperoxy-benzoic acid	1964	0.5	2/114	57/11.4	C	10	9	9	9	9	8	23		
	<i>p</i> -Nitroperoxy-benzoic acid	1964	0.05	2/114	5.7/11.4	C	10	8	6	6	5	5	4, 7, 8		
	<i>p</i> -Nitroperoxy-benzoic acid	1965	0.05	2/104	5.2/10.4	C	15	11	11	10	9	8	4		
	<i>p</i> -Nitroperoxy-benzoic acid	1967	1.0	1/26	26/2.6	S	16	7	7	6	5	4			
	<i>p</i> -Nitroperoxy-benzoic acid	1967	0.05	1/26	1.3/2.6	S	16	16	16	15	12		15	16	L(9), Sa(16, 16)
XXVIII	Peroxy-lauric 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			

*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.

involved 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	Other tumors
<i>Controls</i>								
	Tricaprylin	S ^a and C	104	1	5	2	0	
	No treatment	S and C	202	1	11	14	4	
<i>Fatty acids and esters</i>								
I	Lauric acid	S	28	1	2	0	2	
II	Palmitic acid	S	26	2	2	3	1	
III	Oleic acid	S	26	0	0	1	0	
IV	Stearic acid	S and C	83	4	3	0	1	
V	4-Ketostearic acid	C	13	2	0	0	0	
VI	2-Hydroxystearic acid	C	14	0	2	0	0	
VII	12-Hydroxystearic acid	S	28	9	2	0	2	
VIII	Methyl stearate	S	32	2	2	0	0	
VIX	Methyl 12-hydroxystearate	S	27	8	2	1	0	
X	9(10)-Monohydroxystearic acid	C	13	1	1	0	0	
XI	Stearohydroxamic acid	C	14	3	5	0	2	
<i>Lactones</i>								
XII	β -Butyrolactone	S	15	1	0	0	0	0
XIII	γ -Butyrolactone	S	14	0	0	0	0	0
XIV	α -Angelicalactone	S	15	0	0	0	0	0
XV	γ -Valerolactone	S	16	0	0	0	0	0
XVI	γ -Heptalactone	S	15	0	0	0	0	0
XVII	2,2,4-Trimethyl-3-hydroxy-3-pentenoic acid lactone	S	16	0	0	0	0	0
XVIII	γ -Octalactone	S	16	1	0	0	0	0
XIX	γ -Nonalactone	S	15	1	0	0	0	0
XX	γ -Undecalactone	S	4	0	0	0	0	0
XXI	γ -Stearolactone	S	117	15	11	0	0	3
<i>Epoxy compounds</i>								
XXII	<i>cis</i> -9,10-Epoxyoctadecanoic acid	S and C	70	6	5	1	1	4
XXIII	<i>trans</i> -9,10-Epoxyoctadecanoic acid	C	14	1	0	0	0	0
XXIV	Glycidyl laurate	S and C	30	15	7	0	0	1
XXV	Glycidyl oleate	S and C	13	5	4	0	1	0
XXVI	Glycidyl stearate	S and C	44	3	5	1	0	0
<i>Peroxy compounds</i>								
XXVII	<i>p</i> -Nitroperoxybenzoic acid	S and C	59	6	1	0	2	2
XXVIII	Peroxy lauric acid	S	13	0	1	1	3	0
XXIX	Lauryl peroxide	C	15	0	0	0	0	0

^aS, 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 16 of 306 mice surviving 6 months (5.2%). Among 262 virgin Swiss 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 38 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 10 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-Epoxyoctadecanoic acid (XXIII) 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 epoxyoctadecanoic 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
<i>p</i> -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
<i>p</i> -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 unreproducible results, was critically viewed by Arfman (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 the 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 (XIII), 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 subcutaneous injection in mice. The mice were selected because with most 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 coreaction 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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Brain Nerve Cell Tumors in Mice on Diets Supplemented with Various Lipids¹

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Egg fat, which was found to cause a high incidence of various types of malignant tumors in mice [3], is very rich in cholesterol and lecithin. The present experiments were carried out in order to find out whether the carcinogenicity of eggs is due to either of the above two lipids or to the glycerides.

Materials and Methods

Mice of the T.M. strain were placed at the age of 4 weeks on the Purina mice chow (Group 1, control) supplemented with the following lipids: 2. refined corn oil (which is free of phospholipids); 3. refined corn oil and cholesterol; 4. monoolein (a non-saturated glyceride); 5. monoolein and cholesterol; 6. monostearin (a saturated glyceride); 7. monostearin and cholesterol; 8. vegetable lecithin; 9. lecithin and cholesterol; and 10. cholesterol alone. The mice of the 10 groups were bred and their offspring maintained on the same diets. Most of the mice were, consequently, on the above diets from the day they were born. The Purina chow was available to the mice at all times, while the various lipids mixed with some sugar (so that the mice eat them) were given to them once daily in the following amounts: refined corn oil 100 to 150 mg, the glycerides 50 to 100 mg, lecithin 5 to 10 mg and cholesterol 4 to 5 mg/mouse daily. The mice were kept until they died or became moribund and were killed. The incidence of the different types of malignant tumors varied with the type of lipid consumed by the mice, it was not the same as that obtained with egg lipids. The results of these experiments will be described in the near future. The present is a report on the brain nerve cell tumors found in many of the experimental mice.

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Results

Of the mice on the diets supplemented with the various lipids 95 developed brain tumors varying in diameter from 2 to 5 mm, except for three of them which were close to 1 cm in diameter and extended over both hemispheres. All of these tumors projected from the upper surface of the frontal lobe of the right or left hemisphere (fig. 1 and 2). Many mice had 2 or 3 such tumors. Macroscopically, these tumors limited by a groove, appeared paler in color than the surrounding cortex. The mice with brain tumors at the anterior pole of the hemispheres, just above the entrance of the olfactory tract (fig. 2), were quiet, almost immobile, while those with the tumors at the central or posterior part of the frontal lobe (fig. 1) were excitable and presented very often rotatory movements. The age of the mice varied from 409 to over 900 days, except for one which had a brain tumors and was killed at the age of 261 days.

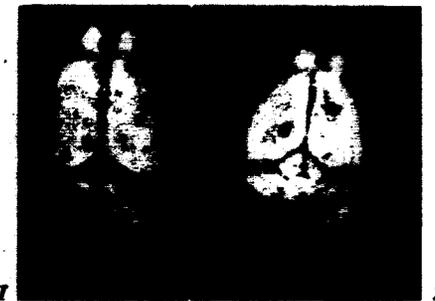


Fig. 1. Brain of a male mouse, 656 days old, maintained on Purina mice chow supplemented with lecithin and cholesterol. $\times 1.5$. The frontal lobe of the right hemisphere has a tumor in its middle.

Fig. 2. Brain of a female mouse 563 days old, maintained on Purina mice chow supplemented with cholesterol. $\times 1.8$. The frontal lobe of the right hemisphere has a tumor just above the entrance of the olfactory tract.

For microscopic studies, some brains were fixed with Bouin's fluid and stained with hematoxylin and eosin (fig. 6), trichromic stain, or with thionin for Nissl's bodies (fig. 7), others were fixed with formaldehyde or with alcohol and impregnated with silver nitrate according to modifications of Bielschowsky's and Cajal's methods (fig. 5). Still others were fixed with formaldehyde and

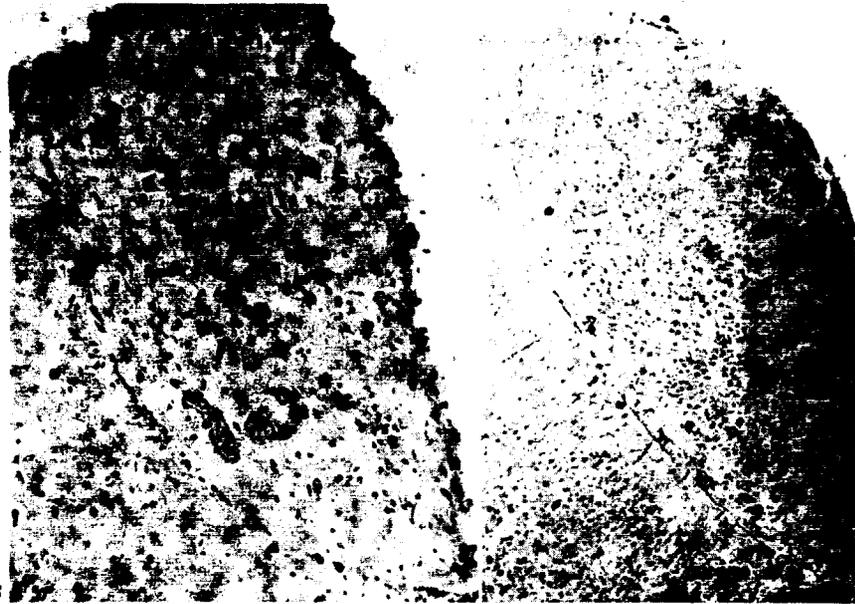


Fig. 3. Section of a brain nerve cell tumor of a female mouse 635 days old, maintained on a diet supplemented with corn oil and cholesterol. Thionin. $\times 200$. A curved line delimits the tumor, to the right, from the molecular layer of the hemisphere, to the left and below. The tumor consists of cells of various sizes and shapes.

Fig. 4. Section of a brain nerve cell tumor of a female mouse, 589 days old, maintained on Purina mice chow supplemented with cholesterol. Cajal's gold sublimate method for neuroglia. $\times 50$. The oblique line from left to right and downward marks the limit between the tumor, which is to the right and the normal cortex, below and to the left. A few neuroglia cells can be seen just above the line of demarcation.

monium bromide and stained with Cajal's gold sublimate for the neuroglia cells (fig. 4).

It was found that all the brain tumors consist almost exclusively of nerve cells. They stain less intensely with eosin than the surrounding cortex. The cells are provided with large nuclei and prominent nucleoli, there were no mitotic figures in these tumors, but binucleated cells were occasionally found (fig. 8). The cytoplasm of these cells extending into many processes contains Nissl's bodies (fig. 3 and 7) and neurofibrils. The latter extend into the cell processes (fig. 5 and 9). In many tumors some of the processes form a bundle of fibers at the boundary with the



Fig. 5. Section of a brain nerve cell tumor of a female mouse 617 days old on Purina mice chow supplemented with lecithin, Cajal's reduced silver method. $\times 50$. The tumor, above and to the left, is separated from the molecular layer of the hemisphere by a well marked oblique line.

Fig. 6. Section of a brain nerve cell tumor of a male mouse, 760 days old, maintained on Purina mice chow supplemented with cholesterol. H. and E. $\times 50$. The tumor is above and to the left of the molecular layer of the hemisphere.



Fig. 7. Nerve cells of the tumor of figure 3 at a high magnification, showing Nissl's bodies in their cytoplasm ($\times 1000$).



Fig. 8. Binucleated cells of the tumor of figure 6 at high magnification ($\times 1000$).



Fig. 9. Typical nerve cell, with processes of figure 5 at high magnification ($\times 500$).

normal cortex without entering the latter (fig. 5). In others, particularly in those originating from the most posterior part of the frontal lobe the bundles of fibers enter the normal cortex. In preparations stained with Cajal's gold sublimate a few neuroglia cells were found at the periphery of the tumors (fig. 4). They form with their processes some kind of a limiting membrane between the tumor and the surrounding cortex. Some of these neuroglia cells have the characteristics of oligodendroglia and others are astrocytes with typical foot plates in contact with blood vessels.

TABLE I
Brain nerve cell tumors in mice of the T.M. strain on diets supplemented with various lipids

Diet	Control	Cholesterol	Corn oil	Corn oil and Cholesterol	Monolein	Monolein and Cholesterol	Monostearin	Monostearin and Cholesterol	Lecithin	Lecithin and Cholesterol
Total number of mice	360	212	159	106	144	158	115	140	166	212
Number of mice brain examined	188	80	11	22	63	64	53	72	73	88
Number of mice with brain tumors	0	20 (25%)	1 (9.1%)	7 (31.8%)	3 (4.7%)	7 (10.9%)	3 (5.6%)	10 (13.8%)	18 (24.6%)	27 (30.6%)

The mice of which the brain was examined were killed when they started to loose weight. Those of which the brain was not examined died at night and by the time they were autopsied it was impossible to remove the brain undamaged.

The incidence of the brain nerve cell tumors was hard to determine. A considerable number of mice died at night and by the time they were autopsied the brain was too soft to be dissected out and examined. The largest number of mice with brain tumors was on the diets supplemented with lecithin, cholesterol, or cholesterol in combination with some other lipid. Of the mice on the diets supplemented with the glycerides alone only 6 developed such tumors. So far, none of the control group developed brain nerve cell tumors (table I).

Discussion

The mechanism by which the lipids influenced the development of brain nerve cell tumors is not known. Russell [2] implanted into the hemispheres of rats methyleholanthrene pellets and obtained, among other neoplasms, two brain nerve cell tumors. He thought that the carcinogen elicited growth of some primitive immature cells which underwent differentiation secondarily. In the present experiments no carcinogen was used, it is suggested, however, that the mechanism of the brain nerve cell tumor formation may be the same. Methylcholanthrene, which is fat soluble, causes probably important changes in the cellular lipid metabolism. The administration over a long period of time, beginning at a very early age, of isolated lipids, may have the same effect upon the primitive nerve cells of the brain. Recently *Strooband and Brucher* [1] obtained 2 nerve cell tumors in rats to which they administered methylnitrosourea by the digestive route.

The reason why these brain tumors appear only on the frontal lobe may be as follows: 1. This lobe undergoes differentiation later than the other parts of the hemisphere and consequently has a large number of primitive cells exposed to the effect of the dietary lipids. 2. There is some connection between the changes in the pituitary and the brain tumors. In the pituitary of the mice on the diets supplemented with lipids there is a decrease in the relative number of acidophile cells and an increase in basophile cells and occasionally development of tumors [4, 5]. The pituitary is known to be influenced in most of its functions by the hypothalamus; and the latter receives most of its cortical afferent fibers from the frontal lobe.

Summary

Mice of the T.M. strain have been maintained on Purina mice chow supplemented with small amounts of various lipids. Ninety five of these mice developed tumors on the upper surface of the frontal lobe. The largest number of mice with brain tumors were on the diets supplemented with lecithin, cholesterol or cholesterol in combination with any other lipid. The tumors consisted mainly of differentiated nerve cells containing Nissl's bodies and neurofibrils.

Acknowledgement

Some of the slides were examined by Dr. *L. J. Rubinstein*, Professor of Neuropathology at Stanford University School of Medicine, to whom I am greatly indebted. Dr. *Rubinstein* classifies the nerve cell tumors as gangli-neuromas.

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360 THE EFFECT UPON MICE OF A DIET SUPPLEMENTED WITH REFINED CORN OIL. J. Szepsenwol, Dept. of Anatomy, Univ. of Puerto Rico School of Medicine.

Mice maintained on a diet supplemented with egg lipids develop a high incidence of lung tumors, lymphosarcomas and mammary cancer. In the present experiments mice of the T.M. strain were placed at the age of 4 weeks on the Furina mice chow (Group 1, control) supplemented with refined corn oil (Group 2) and with corn oil and cholesterol (Group 3). The mice were bred and their offspring maintained on the same diets, so as to have mice of several generations on the above diets from the day they were born. Each mouse received daily 100 to 150 mg. of corn oil (Group 2) and 4 to 5 mg. of cholesterol (Group 3). The purpose of these experiments was to find out whether refined corn oil, free of phytosterols and of cholesterol (plant sterols are sitosterol and stigmasterol) would have the same effect upon mice as egg fat. The results were as follows: The mice of groups 2 and 3 developed a relatively high incidence of lung tumors, while the incidence of mammary cancer was 5% in Group 2 and 10.7% in Group 3. Lymphosarcoma, which was completely absent from Group 2, occurred in 3 out of 130 mice of Group 3. The lymphoid organs in most of the experimental mice were atrophic and depleted of lymphocytes. The mammary tumors occurring in these mice, unlike in those receiving egg lipids, were atypical, they resembled the human ductal type of neoplasm. (Supported by General Research Grant FR 5419).

THE BIOLOGICAL VALUE OF OILS AND FATS

I. GROWTH AND FOOD INTAKE ON FEEDING WITH NATURAL OILS AND FATS

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It has been shown by different investigators that the growth of young rats fed butter-containing diets is better than if the butter is replaced by certain vegetable oils or fats (Schantz et al. ['40], using maize, soybean, cottonseed and coconut; Harris and Rosenfeld ['40], who used coconut and olive oil; Boer ['41], who employed olive and rapeseed oils; Freeman and Ivy ['42], coconut; Parrish et al. ['46], maize; Deuel et al. ['48], rapeseed oil; Euler et al. ['48], rapeseed oil; Nieman et al. ['52], groundnut oil).

In contradiction to these results a number of workers report that no superior growth could be observed in rats fed butter-containing diets (Deuel et al. ['44; '49], maize, cottonseed, olive, groundnut and soybean oil; Henry et al. ['45], groundnut, maize, cottonseed and soybean oil; Zialcita and Mitchell ['45], maize oil; Lassen and Bacon ['49], olive and cottonseed oil).

In the present investigation the growth-action of a large number of fats and oils has been studied. Since previous experiments had shown that the growth depends on the fat-content in the diet (Hoagland and Snider ['40; '41], Boutwell et al. ['43], Forbes et al. ['46], Barki et al. ['50]), each fat was investigated at different dosage levels, varying from 10 to 73 Cal. %.

EXPERIMENTAL

For each fat a series of 8 groups, each consisting of 8 to 12 male 21-day-old Wistar-strain rats, was used. Occasionally the number of groups was smaller than 8. All groups belonging to the same test fat series were started simultaneously, but each test fat was investigated at a different point of time.

Seven of the 8 groups received a diet in which the test fat was mixed in amounts of 10, 20, 30, 40, 50, 60 and 73 Cal. %, respectively. The 8th group served as a reference standard; its diet contained 20 Cal. % of summer butterfat. The com-

TABLE 1
Composition of the diets

DIET	RICE	CASEIN	YEAST	SALTS	BUTTER FAT	TEST FAT	CALORIES PER GRAM
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	
C20-Summer butterfat	157.6	34.5	28.5	8.5	21.5	...	3.99
C10-test fat	187.8	32.1	28.5	8.5	...	10.8	3.74
C20-test fat	157.6	34.5	28.5	8.5	...	21.5	3.99
C30-test fat	127.4	36.9	28.5	8.5	...	32.3	4.28
C40-test fat	97.2	39.4	28.5	8.5	...	43.0	4.62
C50-test fat	67.0	41.8	28.5	8.5	...	53.8	5.01
C60-test fat	36.8	44.2	28.5	8.5	...	64.5	5.48
C73-test fat	...	47.2	28.5	8.5	...	77.6	6.18

position of the diets used for the investigation of a given test fat is recorded in table 1.

All of the diets contained the same amounts of protein, water-soluble vitamins (dried brewers' yeast), and salts per calorie unit; e.g. 230 Cal. protein, 28.5 gm yeast, and 8.5 gm salt-mixture per 1000 Cal. The content of test fat in the diets was varied at the expense of the carbohydrate content on a caloric base. Food and drinking water were given ad libitum; the diets were prepared twice a week. The fat-soluble vitamins were administered in the form of a prophylactic dose. At the beginning of the experiment and again after 4 weeks the animals were fed by pipette 0.05 ml of a concen-

trate containing 2000 I.U. vitamin A, 12.5 μ g vitamin D₂, and 10 mg tocopherol-acetate, dissolved in groundnut oil. The animals were weighed weekly in the course of the 6-week experiment. The quantity of food supplied was also determined, but the wastage was not taken into account.

RESULTS

Twenty different oils and fats have been studied. Two of them, e.g. butter and whale oil, were investigated in duplicate. The results of the increase in weight after one, three and 6 weeks are shown in table 2.

The data obtained with the two summer-butterfat groups show that the rate of growth remains practically unchanged when the fat content of the diets is increased. Only in the first few weeks and at the highest dosage, viz. 73 Cal. % summer butterfat, is the growth somewhat less than at the lower dosages; but at the end of the experiment this difference has disappeared. With the other fats, however, with the exception of lard, there was, to a greater or lesser extent, a decrease of the rate of growth with increasing fat dosages.

In order to compare the growth-promoting effect of various fats with each other and in particular with summer butterfat, the difference between a test group and the simultaneously started standard group (20 Cal. % summer butterfat) was considered a suitable criterion. The comparison with butterfat was facilitated by deducting the mean value of the two butterfat series from the differences obtained. The growth-promoting value of summer butterfat was thus zero.

In table 3 a survey is given of the results obtained after statistical treatment of the experimental data. Only the observations after three weeks are recorded. The order in which the various fats are placed is determined by the magnitude of the differences as compared with summer butterfat. Since the growth of the rats in the butterfat groups after three weeks is about 100 gm, the numerical values in the table represent approximate percentages.

TABLE 2
 Mean increase in weight after 1, 3 and 6 weeks (gm)

CAL. %	LARD				SUMMER BUTTERFAT I				SUMMER BUTTERFAT II			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	24	99	195	12	27	107	206	12	25	100	196
20	10	22	100	199
30	12	25	109	216	12	26	109	218	12	25	104	211
40	12	25	107	211	12	27	108	211	12	25	105	206
50	12	25	105	218	12	25	111	220	12	24	105	218
60	12	24	104	220	12	26	110	211	12	24	103	217
73	12	23	105	220	12	21	103	216	12	21	99	208
Standard ¹	12	22	97	194	12	28	111	212	12	25	104	205

CAL. %	OLIVE OIL				COTTONSEED OIL				WINTER BUTTERFAT			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	23	101	202	12	20	79	153
20	12	24	103	201	11	21	98	202	12	19	81	171
30	12	24	103	209	12	19	82	171
40	12	22	96	195	12	24	107	217	12	15	80	176
50	12	22	97	204	12	18	78	170
60	12	19	99	203	11	21	99	213	12	14	78	177
73	12	17	90	185	12	17	87	196	12	13	70	181
Standard ¹	12	24	102	195	12	24	101	209	12	18	80	166

CAL. %	BEEF FAT				SHEA BUTTER				MAIZE OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	28	108	208	12	25	95	179	9	21	93	188
20	10	26	105	196	12	24	95	178	9	23	98	199
30	12	28	108	210	12	22	89	169	9	22	94	192
40	12	26	105	202	12	21	90	171	9	23	99	202
50	12	27	106	210	12	20	87	169	9	25	104	216
60	12	27	106	209	12	20	89	169	9	21	92	195
73	12	18	93	201	12	19	88	174	8	16	74	181
Standard ¹	9	28	111	213	12	22	93	181	10	22	94	189

CAL. %	SOYABEAN OIL				GROUNDNUT OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	11	26	102	190	10	25	107	214
20	11	26	100	184	10	24	104	215
30	11	26	103	198	10	25	109	219
40	11	24	92	184	9	22	102	210
50	11	24	95	188	10	22	105	215
60	11	23	97	200	10	21	99	210
73	11	17	82	184	10	14	84	193
Standard ¹	10	25	102	192	10	24	106	219

¹ Twenty Cal. % summer butterfat.

TABLE 2 (continued)
Mean increase in weight after 1, 3 and 6 weeks (gm)

CAL. %	PALM FAT				SUNFLOWERSEED OIL				POPPYSEED OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	9	27	106	204	11	26	102	204	11	24	98	197
20	9	28	104	203	12	26	103	208	11	24	98	199
30	9	30	110	207	11	25	101	208	12	24	101	207
40	9	29	110	212	12	24	103	207	11	24	99	202
50	8	27	107	214	12	24	101	214	11	24	101	207
60	8	23	100	197	10	21	88	191	11	20	89	200
73	9	16	91	193	12	17	75	177	11	13	69	177
Standard ¹	9	29	111	218	12	26	105	204	11	25	104	208

CAL. %	COCONUT FAT				SESAME OIL				OWALANUT OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	25	98	196	11	22	89	179	12	23	96	...
20	12	23	95	195	10	24	97	192	12	21	89	...
30	12	24	98	197	11	21	90	186	12	22	92	...
40	12	22	92	191	10	20	86	183	12	18	85	...
50	12	21	92	198	11	19	81	182	12	18	81	...
60	11	18	83	182	11	19	76	173	12	15	75	...
73	11	13	65	156	9	10	49	139	12	6	31	...
Standard ¹	12	26	102	203	11	22	91	190	12	22	94	...

CAL. %	WHALE OIL I				WHALE OIL II				HERRING OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	11	25	99	187	10	24	97	201	12	29	107	209
20	12	23	85	169	10	20	84	181	12	23	96	189
30	11	22	85	168	10	20	85	178	12	22	90	180
40	11	20	82	163	10	20	80	177	12	19	82	164
50	11	19	74	141	10	18	76	175	12	14	70	151
60	12	14	61	149	11	13	67	168	12	12	60	127
73	11	6	47	135	8	6	48	158	12	5	44	104
Standard ¹	11	23	97	186	10	24	100	214	12	29	108	212

CAL. %	RAPESEED OIL				KAPOKSEED OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	8	21	93	183	12	16	98	187
20	10	22	81	154	12	15	73	169
30	9	19	75	149	11	12	57	142
40	9	13	56	131	12			
50	9	12	52	125	12	{ 7 animals died (mean 18 days) 9 animals died (mean 15 days) all animals died (mean 14 days)		
60	9	7	35	96	12			
73	9	(all animals died, mean 17 days)			11			
Standard ¹	9	24	94	177	11	24	104	212

¹ Twenty Cal. % summer butterfat.

Table 3 shows that the difference between growth with summer butterfat and the other oils and fats increases with increasing fat content in the diet. Since the growth-promoting effect of summer butterfat is practically independent of the dosage, the augmentation of the difference is actually due to a lowering of the rate of growth caused by the different oils and fats when their dosage is increased.

TABLE 3
Difference in growth-promoting value (gm) as compared with
summer butterfat (3rd week)

GROUP	TYPE OF FAT	CALORIC PERCENTAGE TEST FAT IN THE DIET						
		10	20	30	40	50	60	73
1	Lard	+ 5	+ 3	+ 12	+ 10	+ 7	+ 7	+ 14
2	Summer butterfat (Av.)	0	0	0	0	0	0	0
3	Olive oil	-	+ 1	-	- 6	-	- 4	- 6
	Cottonseed oil	+ 4	- 3	+ 3	+ 7	- 5	- 1	- 8
	Winter butterfat	0	- 1	0	- 2	- 5	- 4	- 6
	Beef fat	+ 3	- 3	+ 1	- 3	- 3	- 2	- 10
	Shea butter	+ 3	- 1	- 6	- 5	- 9	- 6	- 2
4	Maize oil	+ 2	+ 4	0	+ 5	+ 8	- 2	- 15
	Soybean oil	+ 5	0	+ 3	- 9	- 6	- 3	- 12
	Groundnut oil	+ 3	- 3	+ 2	- 6	- 4	- 8	- 18
	Palm fat	- 1	- 6	0	0	- 4	- 9	- 13
5	Sunflowerseed oil	0	- 2	- 4	- 2	- 5	- 17	- 24
	Poppysced oil	- 2	- 5	- 2	- 4	- 4	- 14	- 29
	Coconut fat	0	- 5	- 2	- 9	- 9	- 17	- 30
6	Sesame oil	- 2	+ 2	- 4	- 8	- 15	- 18	- 50
	Owalanut oil	+ 5	- 5	- 2	- 9	- 14	- 19	- 58
7	Whale oil (Av.)	+ 3	- 14	- 13	- 17	- 24	- 35	- 45
	Herring oil	+ 1	- 12	- 19	- 27	- 40	- 48	- 59
	Rapeseed oil	- 3	- 18	- 24	- 44	- 49	- 64	†
8	Kapokseed oil	- 13	- 31	- 46	†	†	†	†

From a statistical analysis¹ of the figures it was found that, except for random sampling variations, the variability of the increase in weight after three weeks was the same for all groups. This quantity appears to be independent of the growth rate, the season, and the number in the litter. The best estimate for the variance of the increase in weight in the third week, derived from 1983 observations was found to be $\sigma^2 = 118.1911$. Moreover the analysis showed that the distribution may be considered normal. The values in table 3 which differ significantly ($P = 0.05$) from those of the corresponding summer-butterfat group are boxed in.

In table 3 the various oils and fats are grouped according to the results of the significance test.

This classification of the different oils and fats is fairly artificial and is of value only for purpose of orientation. The fats of group 3, for example, do not differ significantly from summer butterfat, but they were grouped separately, since the differences with respect to summer butterfat at dosages of 50, 60, and 73 Cal. % all have a negative sign. Moreover, their total mean at a dosage of 73 Cal. % differs significantly from summer butterfat.

The further analysis of the gain data concerns the type of the growth curve. It has been found that the increase in weight when the standard diet with 20 Cal. % summer butterfat is administered is practically linear for the first 6 weeks. The growth index is $\frac{\text{increase in weight 3-6 weeks}}{\text{increase in weight 0-3 weeks}} = 0.985$ for a series of 37 standard groups.

The standard error of this mean = 0.092, so that the fiducial limits for $P = 0.05$ are: 0.789 and 1.151.

With the aid of these values it is possible to establish which growth indices deviate significantly from the normal standard (20 Cal. % summer butterfat). Table 4 records these growth indices. The values which deviate significantly are italicized.

¹The statistical problems were studied in collaboration with the "Mathematisch Centrum," Amsterdam. In this connection I wish to thank especially Professor Dr. J. Hemelrijk and his co-workers.

TABLE 4
Growth indices and efficiency constants

TYPE OF FAT	GROWTH INDICES $\left(\frac{\text{INCREASE IN WEIGHT 3-6 WEEKS}}{\text{INCREASE IN WEIGHT 0-3 WEEKS}} \right)$								EFFICIENCY CONSTANTS $\left(\frac{\text{INCREASE IN WEIGHT IN 6 WEEKS}}{\text{FOOD CALORIES IN 6 WEEKS}} \times 100 \right)$								
	Stand-ard (20 Cal. %) summer butter- fat	Caloric percentage test fat in the diet							Stand-ard (20 Cal. %) summer butter- fat	Caloric percentage test fat in the diet							
		10	20	30	40	50	60	73		10	20	30	40	50	60	73	mean
Lard	1.09	0.97	0.99	0.98	0.97	1.08	1.12	1.10	7.13	7.84	7.65	7.56	6.71	7.46	7.07	6.90	7.29
Summer butterfat I	0.91	0.93	0.91	1.00	0.95	0.98	0.92	1.10	8.21	9.80	8.21	8.23	8.25	8.55	7.60	8.10	8.37
II	1.01	0.96	1.01	1.03	0.96	1.08	1.11	1.10	8.24	7.52	8.24	7.73	8.25	7.64	7.89	8.00	7.94
Olive oil	0.91	...	0.95	...	1.03	...	1.05	1.06
Cottonseed oil	1.05	1.00	1.06	1.03	1.03	1.10	1.15	1.25	8.14	8.00	7.68	8.94	8.53	8.80	8.29	8.32	8.34
Winter butterfat	1.08	0.94	1.11	1.09	1.20	1.18	1.27	1.59	6.29	6.69	6.33	6.90	6.40	7.15	6.91	8.15	6.85
Beef fat	0.92	0.93	0.87	0.94	0.92	0.98	0.97	1.16	7.54	8.36	7.13	9.24	7.71	7.24	6.89	7.40	7.69
Shea butter	0.95	0.88	0.87	0.90	0.90	0.94	0.90	0.98	6.40	7.10	6.20	6.32	5.41	5.94	6.01	7.15	6.32
Maize oil	1.01	1.02	1.03	1.04	1.04	1.08	1.12	1.45	7.94	8.70	8.52	8.54	8.93	9.09	8.83	8.52	8.64
Soybean oil	0.88	0.86	0.84	0.92	1.00	0.98	1.06	1.24	7.58	8.02	6.97	8.52	7.37	8.01	8.32	8.25	7.88
Groundnut oil	1.07	1.00	1.07	1.01	1.06	1.05	1.12	1.30	8.23	8.82	8.50	9.05	8.43	8.26	8.76	8.51	8.57
Palm fat	0.96	0.92	0.95	0.88	0.93	1.00	0.97	1.12	9.20	9.21	9.25	9.42	8.43	9.33	7.85	8.63	8.92
Sunflowerseed oil	0.94	1.00	1.02	1.06	1.01	1.12	1.17	1.36	8.13	7.39	8.43	8.74	9.33	7.50	7.95	8.17	8.21
Poppyseed oil	1.00	1.01	1.03	1.05	1.04	1.05	1.25	1.57	8.24	8.36	8.14	9.17	7.81	8.44	9.10	8.53	8.47
Coconut fat	0.99	1.00	1.05	1.01	1.08	1.15	1.19	1.40	8.31	8.20	8.04	8.83	7.50	8.14	7.81	8.07	8.11
Sesame oil	1.08	1.00	0.99	1.08	1.13	1.24	1.27	1.84	6.82	6.85	7.02	7.24	7.40	7.59	7.28	7.48	7.21
Owalanut oil
Whale oil I	0.91	0.89	0.99	0.98	0.99	0.91	1.44	1.87	7.38	7.36	7.84	7.09	7.86	8.33	7.64
II	1.14	1.07	1.15	1.09	1.21	1.30	1.51	2.29	8.34	8.33	7.67	7.88	7.87	7.83	8.22	...	8.02
Herring oil	0.96	0.95	0.97	1.00	1.00	1.16	1.12	1.36	7.55	8.22	6.99	7.68	6.55	7.20	6.58	6.72	7.19
Rapeseed oil	0.88	0.97	0.90	0.99	1.34	1.40	1.74	...	7.30	7.48	6.45	6.82	7.37	7.72	6.91	...	7.15
Kapokseed oil	1.04	1.13	1.32	1.49	7.67	8.03	7.42	7.26	7.60
Mean	0.985 ± 0.092	7.73 ± 0.71	8.05	7.62	8.07	7.69	7.84	7.69	7.95	7.83

Table 4 shows that a number of growth indices differ significantly from the standard, all these deviating values being too large. The data indicate that the deviation from the standard is greater as the rate of growth is smaller. The correlation between the increase in weight in the first three weeks and the magnitude of the growth indices appears to be highly significant ($r = -0.70$). Thus the type of the growth curve is generally linear (growth index = ± 1), but in the case of certain oils and fats with little growth-promoting action, given in a large dose, the rate of growth in the second half of the experiment is better than in the first half. A certain "adaptation" manifests itself, which becomes more pronounced as the growth rate of the animals is diminished.

The data concerning the food intake refer to the total amount of food supplied in 6 weeks to each group. As already mentioned, the wastage of food, which will certainly have occurred and to a varying extent for the different groups, is not taken into account. As was to be expected, there is a positive correlation between the food intake and the increase in weight. For this reason the efficiency constants were calculated. Efficiency constant = $\frac{\text{increase in weight in 6 weeks}}{\text{food calories in 6 weeks}} \times 100$. The second part of table 4 records these efficiency constants. The average of the efficiency constants of the standard groups (20 Cal. % summer butterfat) is 7.73 with S.D. = 0.71. Values which differ significantly from this standard mean are in italics. With the exception of shea butter (too low) and palm oil (too high) the table suggests that the efficiency constants are approximately the same for all the fats and oils. It seems therefore that the increase in weight per calorie of food intake is independent of the type of fat and the level at which it is administered.

Finally, some data are given on the mortality observed in some of the experimental groups. Of the group which received the diet with 73 Cal. % of rapeseed oil all animals died, after 8, 10, 12, 14, 15, 20, 20 and 37 days, respectively (mean 17 days).

- The mortality in the kapokseed oil groups was as follows:
- 40 Cal. %: 7 of the 11 animals died after 6, 14, 18, 18, 21, 21 and 27 days, respectively (mean 18 days).
 - 50 Cal. %: 9 of the 12 animals died after 11, 11, 12, 15, 16, 16, 17, 19 and 20 days, respectively (mean 15 days).
 - 60 Cal. %: all animals died after 8, 10, 13, 14, 15, 15, 16, 16, 17, 18, 19 and 21 days, respectively (mean 14 days).
 - 73 Cal. %: all animals died after 8, 9, 11, 12, 12, 12, 13, 14, 15, 15 and 20 days, respectively (mean 13 days).

DISCUSSION

The 20 oils and fats investigated do not all have the same effect on growth, but show certain differences. It is illogical to explain this by the theory of the presence of an unknown "growth-promoting" substance in butter. For in that case the favourable substance should not only occur in butter but also in the other oils and fats, the content diminishing with decreasing growth-promoting effect. As a consequence, on raising the dosage with these oils and fats an improvement of the growth should be expected, while, on the contrary, a decline has been observed. Moreover, the "deficiency," which will develop by depletion of the growth-promoting substance, starts at the beginning of the experiment so that a so-called "run-out time" is missing. Besides, the values of the growth indices of the oils and fats with low growth-promoting effect are higher than 1, which points to a certain improvement where an aggravation could be expected. All these facts argue against the growth-promoting hypothesis.

In contrast to this, a growth-retardation theory in which the presence of "growth-retarding" substances in various oils and fats is assumed, explains both the lowering of the rate of growth on raising the fat content in the diet as well as the growth differences obtained with the various oils and fats investigated. The poorest-acting oils and fats should contain the largest amounts of retarding substance(s) and the "retarding potency" should decrease as the growth-action of the fat improves. This theory has received support in the

finding that erucic acid, which is present to the extent of about 50% in rapeseed oil, was responsible for the poor growth-action of this oil (Thomasson and Bolding, '55).

Nothing is known about the nature of any growth-retarding substance in the other poorly-acting oils and fats. Assuming them to be fatty acids, attention is focused on the long-chain fatty acids with 20 or more carbon atoms. Leaving the degree of unsaturation out of account, owalanut oil contains 23% of 20-carbon atom acid, whale oil and herring oil 20 and 52% respectively of 20- and 22-carbon atom acids, and rapeseed oil 50% of 22-carbon atom acid. On this basis the hypothesis is suggested that fatty acids with 20 or more carbon atoms have an unfavourable influence on the growth of young animals. Unfortunately, the composition of kapokseed oil is not well-known; otherwise further suggestions could possibly be obtained from this quarter.

The results indicate that the various oils and fats, with the exception of shea butter and palm oil, seem to have the same food efficiency in spite of differing values in promoting growth. Therefore, it is probable that irrespective of the kind of fat and the level at which it is administered, the caloric value for the animal is the same; and moreover that the growth-retarding fatty acid will be metabolized to the same extent and in the same way as the non-retarding ones, but that only the *rate* at which metabolism takes place is different for the various fatty acids. The low caloric value of shea butter may be a result of the high content of unsaponifiable matter (5 to 7%). The cause of the high value of the efficiency constant of palm oil is not known.

The fact that the food intake of the rat can be regulated by means of the type of fat opens up the possibility of a practical application in medicine, insofar as this rule also holds good for man. In particular, a fat which lowers the food intake will have a chance in the treatment of those forms of obesity which are due to excessive food intake. This problem is under further investigation.

The growth and food intake of the animal form a generally accepted criterion for judging its state of health and a retarded rate of growth is considered as an expression of a pathological condition, or a dysfunction. On this basis the biological value of the oils and fats with a good growth-promoting effect will be rated higher than those with a poor action. This view seems to be supported by the fact that the animals on diets containing high dosages of rapeseed oil or kapokseed oil died within the 6-week experimental period. This conception, however, is incorrect, as has become apparent from a further investigation (Thomasson, '55). It appeared that rats fed 50 Cal. % of rapeseed oil lived significantly longer than rats on a diet containing 50% summer butterfat, although the growth rate, and the daily food intake, of the rapeseed oil animals were much poorer than those of the animals fed butterfat. This observation indicates that growth-inhibiting substances in oils and fats need not of necessity have an adverse effect, but can have a favourable influence instead. In addition, the longer life span observed supports the conception that growth-retarding substances can be useful in the therapy of obesity. Finally, this investigation argues strongly against the existence of an unknown vitamin in butter. For, now that it has appeared that animals receiving this factor — the butter group — have a shorter life span than the "deficient" rapeseed oil animals, the existence of such an unknown butter factor becomes highly dubious.

SUMMARY

In 6-week feeding experiments with rats the behaviour of 20 oils and fats has been investigated, each of them being administered in 7 different dosages from 10 to a maximum of 73 Cal. %.

The various oils and fats showed differences in growth-promoting effect. Nevertheless, the food-efficiency seemed to have a constant value and to be independent of the type of fat used. Only shea butter and palm oil were exceptions to this rule.

As an explanation of the differences observed the presence of growth-inhibiting substances is suggested. Such substances need not be considered as harmful, but may possibly even exert a favourable influence, judging from the data on longevity.

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Toxicity of Saturated Fat^{1,2}

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ABSTRACT When diets containing high levels (20 to 40%) of either palmitate or stearate are fed to weanling mice, poor growth and high mortality result. Adult mice are similarly affected, although less severely. The addition to the diet of 4% of fats rich in either oleate or linoleate prevents the toxicity, whereas linolenic, palmitoleic and petroselinic acids are much less effective. The toxicity is increased slightly by the addition of cholesterol and very markedly when lactose constitutes the principal dietary carbohydrate. Depot fat levels of the dietary saturated fatty acid increase only slightly, particularly when compared with the changes observed when unsaturated fatty acids are fed. Digestibility studies preclude ascribing these effects to poor assimilation of the dietary fat.

The effect of dietary oleate and linoleate on the composition and distribution of fatty acids in the depot fat of mice has been previously investigated (1, 2). When similar studies were attempted with palmitate and stearate, poor growth and high mortality were encountered. Moreover, the depot fat levels of the saturated acids barely increased compared with expectations based on the studies with the unsaturated fatty acids. The results of these investigations provide the basis for the present report.

EXPERIMENTAL

The animals used in these experiments were either weanling or adult male mice obtained from the North Carolina State Laboratory of Hygiene. They were fed a diet containing 20% of casein, 5% of salt mixture W³ and the remaining 75% composed of various mixtures of carbohydrate and fat. The diet was supplemented with all of the known vitamins⁴ (3). The usual duration of an experiment was 3 weeks.

In the experiment in which the digestibility of the fat was determined, 12 adult mice were used. Digestibility was computed from the excretion of chromic oxide over a 5-day experimental period following a 5-day preliminary period. To facilitate the analysis of chromium, the radioisotope Cr⁵¹ was used and radioactivity was measured in a Nuclear-Chicago gamma scintillation spectrometer.

Triglycerides of the depot fat were isolated and their fatty acid composition de-

termined by gas chromatography on a succinate-ethylene glycol polyester column (2).

RESULTS

Effect of saturated fat on weanling mice. Unlike results observed with unsaturated fat, as the dietary level of glycerylmonopalmitate and glycerylmonostearate was increased beyond 10%, there was a progressive decrease in growth and increase in mortality of the experimental animals (table 1). The toxic effect of glycerylmonopalmitate was more severe than glycerylmonostearate. Autopsy of animals that died revealed no gross abnormalities other than that of emaciation. In most instances the adipose tissue was completely devoid of fat, although the stomach and intestines contained considerable quantities of food and the animals continued to eat until the day of death.

Bosshardt et al. (4) reported toxicity of diets containing 20% of palmitic acid, 1% of cholesterol and 0.05 to 0.15% of oleic acid when fed to weanling mice. Omission of either the cholesterol or oleic acid alleviated the toxic effects encountered in the mixture with male mice, whereas 20% of

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TABLE 1
Effect of glycerylmonopalmitate and glycerylmonostearate on growth and mortality of weanling mice¹

Dietary level	Glycerylmonopalmitate		Glycerylmonostearate	
	Wt gain	Mortality	Wt gain	Mortality
%	g	%	g	%
0	2.0	0	1.1	0
5	3.2	0	1.2	0
10	2.1	0	2.1	0
20	-6.2	40	0.9	0
30	-5.6	40	-0.3	0
40	—	100	-1.1	0
50	—	—	-8.6	17

¹ Duration of experiments 3 weeks. Five mice were used for each dietary level of glycerylmonopalmitate and 6 mice for each dietary level of glycerylmonostearate.

palmitic acid alone adversely affected the growth and mortality of weanling female mice. Postmortem examination revealed small spleens as the only gross abnormality. The results of the experiment of table 1 differ from those of Bosshardt et al. (4) chiefly in that the toxic effects of saturated fats on male mice were obtained without the addition of cholesterol, and thus resemble their observations with female mice. The oleum percomorph used as a source of the fat-soluble vitamins could have contributed the trace of oleic acid these investigators (4) found to be required for toxicity.

Effects of unsaturated fats on the toxicity of saturated fat. Although the diets used in this study contained no added source of essential fatty acids (other than the oleum percomorph), no sign of a deficiency of this essential nutrient had appeared in 3 weeks in previous investigations with fat-free diets of similar composition (1-3). However, since the addition of saturated fat to the diet has been shown to promote an essential fatty acid deficiency (5), it appeared possible that the lack of growth and high mortality encountered with the high levels of glycerylmonopalmitate and glycerylmonostearate might represent an acute form of this deficiency disease. On the other hand, Bosshardt et al. (4) had noted that increasing the dietary level of oleic acid reversed the toxicity of their saturated fat diet.

With weanling mice both oleic acid and glycerylmonooleate were as effective as either safflower oil or the fatty acids from safflower oil (table 2) in preventing the

toxicity of the saturated fat. Therefore, the toxicity of the saturated fat is not merely an uncomplicated essential fatty acid deficiency. Since free fatty acids, monoglycerides and triglycerides are all effective, the glyceride form of the unsaturated fatty acid is of no consequence in the prevention of the toxicity of glycerylmonopalmitate. The failure of either polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monooleate (Tween 80) or paraffin oil to effect a reversal of the toxicity of the saturated fat indicates that the action of the unsaturated fats is more than a mere alteration of the physical consistency of the dietary fat.

Although the effects are less pronounced, the diet containing 40% of glycerylmonopalmitate is also toxic to adult mice (table 2). As with weanling mice, the addition of a fat rich in unsaturated fatty acids is effective in preventing the toxicity of the saturated fat in the adult animal. In contrast, supplementation of the diet with coconut oil was less effective, whereas α -tocopheryl acetate gave no protection whatsoever.

In an experiment using free palmitic acid rather than the monoglyceride, the study of the reversal of the toxicity of saturated fat by unsaturated fat was extended to other unsaturated fatty acids. Although the free palmitic acid when fed at a level of 40% proved more toxic than 40% of glycerylmonopalmitate (equivalent to 31% of palmitic acid), the inclusion of 4% of safflower oil in the diet was effective in reversing the toxic effects of the higher intake of saturated fatty acid (table 3)

TABLE 2
Reversal of toxicity of glycerylmonopalmitate by unsaturated fat¹

Diet supplement	No. mice	Wt gain	Mortality
Weanling mice			
None	23	- 0.1	74
4% Safflower oil	17	3.2	6
4% Safflower oil fatty acids	6	4.6	17
4% Oleic acid	6	4.6	0
4% Glycerylmonooleate	11	2.0	18
4% Tween 40 ²	5	—	100
4% Tween 80 ³	6	- 1.4	67
2% Paraffin oil	5	- 1.2	60
Adult mice			
None	6	- 1.9	33
4% Safflower oil	6	0.1	0
4% Corn oil	6	0.1	0
4% Olive oil	6	1.0	17
4% Lard	6	1.0	0
4% Coconut oil	6	- 0.7	17
0.1% α -Tocopheryl acetate	6	- 3.2	50

¹ Duration of experiments 3 weeks. All diets contained 40% of glycerylmonopalmitate.

² Polyoxyethylene sorbitan monopalmitate, Atlas Powder Company, Wilmington, Delaware.

³ Polyoxyethylene sorbitan monooleate.

TABLE 3
Effect of unsaturated fatty acids on the reversal of toxicity of palmitic acid¹

Unsaturated fatty acid	No. mice	Mortality
None	11	100
Linoleic	10	20
Linolenic	12	83
Palmitoleic	10	60
Petroselinic	10	70

¹ Duration of experiments 2 weeks. Diets contained 40% of palmitic acid and 4% of the unsaturated fatty acid. Linoleic acid was fed as safflower oil. Data given are the average of 2 experiments.

In contrast, neither linolenic acid, palmitoleic acid nor petroselinic acid, when added to the diet, was capable of preventing the toxicity of the saturated fat.

The results of a study to ascertain the dietary level of safflower oil required for prevention of the toxicity of glycerylmonopalmitate are shown in figure 1. As the level of safflower oil in the diet increased, there was a decrease in the mortality of the animals and an increase in the growth of those animals that survived. Since 4% of safflower oil was about as effective as 8%, it is apparent that a small quantity of unsaturated fat will alleviate the effects of a tenfold amount of saturated fat.

Effect of cholesterol on the saturated fat toxicity. In the experiments of Bosshardt

et al. (4) with male mice ingesting a diet containing 20% of palmitic acid, the adverse effects caused by the saturated fat were not observed unless cholesterol was included in the diet. Although dietary cholesterol was not required to produce the toxic effects of the saturated fat when the diet contained a higher level of palmitic acid (table 2), it appeared likely that the addition of cholesterol might accentuate the toxic effects obtained with the higher level of saturated fat. The results of a study designed to test this hypothesis (table 4) showed that cholesterol did enhance the toxic effects of palmitate. This is noticed most strikingly in the diets containing 2% of safflower oil where cholesterol addition doubled the mortality and greatly reduced the growth of the animals that survived. When, however, the level of safflower oil was increased to 4%, only a slight difference in growth rate was observed, and even this disappeared when 8% of unsaturated fat was included in the diet.

Effect of carbohydrate on toxicity of saturated fat. When the sucrose component of the diet was replaced by glucose, no effect on the toxicity of the glycerylmonopalmitate was observed (table 5). When, however, lactose was used as the dietary carbohydrate, the toxicity of the

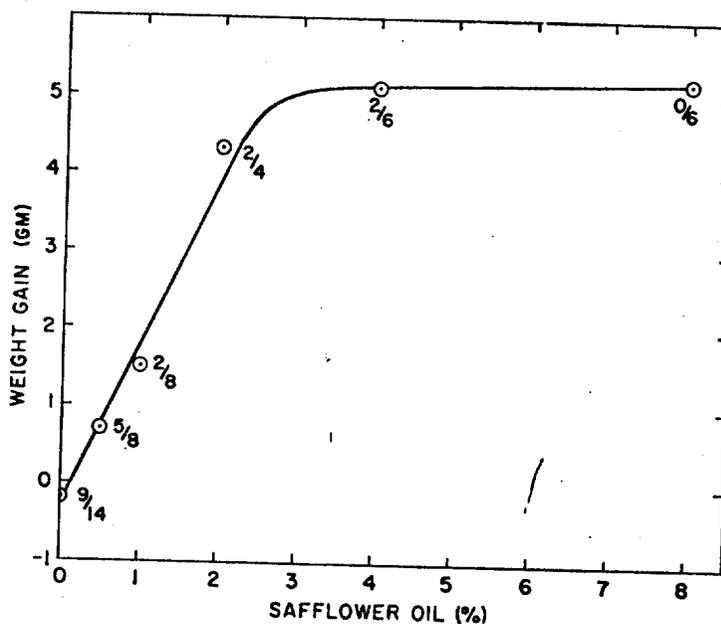


Fig. 1 Effect of safflower oil supplementation on toxicity of glycerylmonopalmitate. Basal diet contained 40% glycerylmonopalmitate. Data are 3-week weight gain of survivors of 2 experiments with weanling male mice. Fractions show mortality and total number of mice for each level.

TABLE 4

Effect of cholesterol on toxicity of glycerylmonopalmitate¹

Safflower oil	Cholesterol			
	None		1%	
	Wt gain	Mortality	Wt gain	Mortality
None	g	%	g	%
2%	—	100	-3.5	83
4%	4.2	33	1.2	67
8%	5.1	33	3.3	33
	5.2	0	5.7	0

¹ Duration of experiment 3 weeks with 6 mice/treatment. All diets contained 40% of glycerylmonopalmitate.

TABLE 5

Effect of carbohydrate on toxicity of glycerylmonopalmitate¹

Carbohydrate	1 Week		3 Weeks	
	Wt gain	Mortality	Wt gain	Mortality
	g	%	g	%
Sucrose	-1.1	25	-0.2	37
Glucose	-0.1	37	2.0	50
Lactose	—	100	—	—

¹ Diets contained 40% of glycerylmonopalmitate and 35% of the carbohydrate indicated. Each diet was fed to 6 mice.

TABLE 6
Toxicity of glycerylmonopalmitate in the presence of lactose

Diet addition	GMP ¹ level	No. mice	Wt gain	Mortality
	%		g	%
None	0	12	1.7	42
None	10	6	5.2	17
None	20	18	4.2	83
None	40	12	—	100
4% Safflower oil	20	12	2.6	25
8% Safflower oil	20	12	4.2	25
8% Safflower oil	40	6	2.6	67
40% Lard	0	6	7.7	0

¹ Glycerylmonopalmitate.

diet became much more acute, diarrhea developed and none of the animals survived the first week of the experiment. Autopsy of these animals disclosed marked intestinal edema, in addition to the emaciation previously observed with the high level of saturated fat.

Because the adipose tissue of animals that ingest lactose becomes depleted of fat (6), as is the case for mice fed diets rich in saturated fat, the interrelationship between these 2 dietary ingredients was investigated further. When a diet containing 35% of lactose and no fat was fed, high mortality and poor growth were observed (table 6). These results are consistent with the observations of other investigators (7). The addition of 10% of glycerylmonopalmitate resulted in marked improvement in both growth and survival. When, however, the level of saturated fat was increased to 20%, a mortality double that encountered with the diet containing no added fat was observed, although the growth of the animals that survived was not different from those fed the diet containing 10% of fat. Finally, when the saturated fat level was increased to 40%, complete mortality was observed before the end of one week. The addition of safflower oil reduced the toxicity of these diets (table 6), but the unsaturated fat was not as effective as in diets when lactose was absent (fig. 1). In contrast with the diets containing high levels of saturated fat, good growth and survival were obtained when the mice were given a diet containing 40% of lard.

Digestibility of saturated fat. It has long been recognized that saturated fats are less digestible than unsaturated fats,

and that increasing the fluidity of a saturated fat by the addition of an unsaturated fat enhances its digestibility (8). In view of the syndrome of the saturated fat toxicity, it appeared possible that caloric insufficiency resulting from the low absorption of the saturated fat might account for the results observed. The rather high digestibility of the saturated fat diets (table 7) leads to a rejection of this hypothesis.

TABLE 7
Food consumption and digestibility of diets containing glycerylmonopalmitate (GMP)

Diet	Consumption	Fat digestibility
	g/mouse/day	%
40% GMP	2.8	81.6
40% GMP + 4% safflower oil	3.1	92.2

Although the addition of 4% of safflower oil increased the absorption of fat, the additional calories absorbed could hardly account for the marked effect of the unsaturated fat on survival and growth. Because of the method of extraction used, it is possible that not all of the fecal lipids were removed and that the fat digestibility figures of table 7 are higher than the true values. If one assumes, however, that the feces consisted entirely of fat, the fat digestibility would be 53 and 66% for the saturated fat diet alone and supplemented with safflower oil, respectively. Therefore, it is clear that mice are capable of absorbing an appreciable amount of the palmitate as well as a major proportion of the energy contained in the diet.

Deposition of saturated fatty acids. It has been long established that when an

animal ingests an unsaturated fat, the unsaturated fatty acids of the depot fat increase proportionately. Indeed, previous studies had shown that dietary oleate and linoleate are deposited in the depot fat of mice (1). It had been the initial objective of this investigation to extend the study of the effect of dietary fat on depot fat to palmitic and stearic acids. Therefore the fatty acid composition of the depot fat of the animals that survived the initial experiments (table 1) was determined by gas chromatography. In contrast with expectations, very little increase in the depot fat level of either palmitate or stearate resulted from the inclusion of the monoglycerides of these fatty acids in the diet (fig. 2).

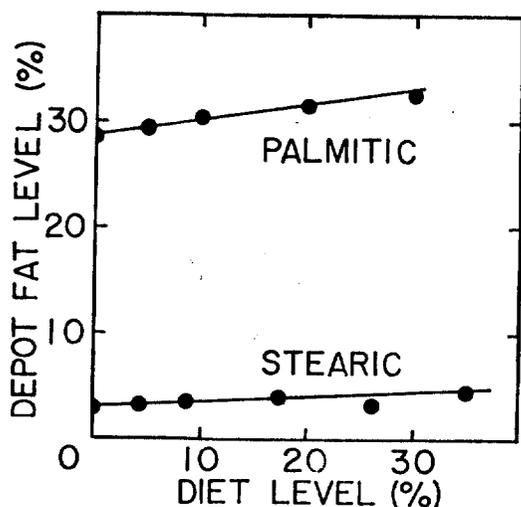


Fig. 2 Effect of dietary palmitic and stearic acids on their levels in depot fat. Fatty acids were fed as the monoglycerides. Data are derived from the survivors of the experiment described in table 1.

The magnitude of the differences between the effect of unsaturated fatty acids and saturated fatty acids on the composition of depot fat can best be illustrated by comparing the slopes of their response curves. The depot fat level of palmitic acid increased 0.06% for each percentage increase of palmitate in the diet, whereas for stearate the slope was only 0.03% (fig. 2). In contrast, for each percentage increase of oleate or linoleate in the diet the percentage in the depot fat increased more than 1 and 2%, respectively (1). These

observations relate to the net change in the level of saturated fatty acids in the depot fat and do not mean that the dietary palmitate and stearate per se are deposited only slightly. The lack of increase in the level of saturated fatty acids could arise equally if the synthesis, or deposition, or both, of these acids from endogenous sources was inhibited by the influx of preformed saturated fatty acids from the diet. An indication that the deposition of endogenous saturated fatty acids may be inhibited is derived from the fact that the deposition of dietary pentadecanoic acid and dietary heptadecanoic acid was observed to be greater than the increase in palmitate deposition, although not as great as the increase obtained when the unsaturated fatty acids were fed.³

DISCUSSION

When high levels of saturated fat were fed to mice, poor growth and high mortality resulted. The only major outward manifestation of this toxicity was emaciation as evidenced by a depletion of the adipose tissue of the mice. This toxic condition can be prevented by the addition to the diet of a relatively small amount of oleate or linoleate. These observations are similar to those of Bosshardt et al. (4) who reported toxicity of diets containing 20% palmitic acid and 1% cholesterol. The latter investigators did not obtain the toxic effects in the absence of cholesterol in male mice but did in female mice. From the data reported herein it is apparent that increasing the dietary level of palmitic acid eliminates the need for cholesterol, although the addition of cholesterol increases the toxicity of these diets.

Adverse effects of feeding diets rich in saturated fatty acids are well established. The addition of saturated fat to diets deficient in linoleic acid decreases the time required for the onset of the essential fatty acid deficiency symptoms and increases their severity (5). Since, however, oleic acid is as effective as linoleic acid in reversing the toxicity of the saturated fat (table 2), the toxicity is not merely an uncomplicated essential fatty acid deficiency. On the other hand, there is some specificity associated with the reversal, in that oleic

³ Tove, S. B., unpublished observations.

acid and linoleic acid are both equally effective, whereas linolenic acid, petroselinic acid and palmitoleic acid are much less effective. Herting et al. (9) noted that a foreign-body type of reaction in fat cells, lipogranuloma, developed when rats were given diets rich in saturated fat. They also observed that the lesions could be prevented by the addition of unsaturated fat to the diet. However, the level required (50% of the total) was much greater than that observed in our experiments (table 2). Although gross examination of the adipose tissue of the mice revealed no sign of the lipogranuloma, it is possible that the saturated fat toxicity reported herein represents an acute form, and the lipogranuloma described by Herting et al. (9), a chronic form of the same metabolic defect.

The mechanism by which the saturated fat produces the toxicity is unknown. The fat depletion of the adipose tissue indicates that the animals were not capable of meeting their energy needs. Yet the mice consume a diet until the day they die, and from the digestibility coefficient it is apparent they are receiving adequate calories from their diet. The high level of saturated fat in the diet may promote uncoupling of oxidative phosphorylation. Oxidative phosphorylation of liver mitochondria is uncoupled in essential fatty acid deficiency (10).

It is generally considered that when any fatty acid is consumed by an animal, that fatty acid increases in the depot fat. This conclusion is based largely on work with unsaturated fatty acids and with short-chain fatty acids such as those in coconut oil. For these fatty acids the statement has been amply proved (11). In contrast, from our results, the addition of palmitate or stearate to the diet does not produce a major increase in the levels of these acids in the adipose tissue. Although saturated fatty acids are not as digestible as unsaturated fatty acids, the digestion coefficients (table 7) indicate that low assimilation cannot entirely account for the failure of

the level of palmitic acid in the depot fat to rise with increased dietary level. The digestibility of glycerylmonostearate, although lower than that of the palmitate (12), also could not entirely account for the low deposition of dietary stearic acid. In this respect, it is of interest to note that it is possible to increase the stearate level of the depot fat of mice more by feeding linoleic acid (1) than by feeding stearic acid.

ACKNOWLEDGMENT

The technical assistance of Mrs. Anne W. Cannon is gratefully acknowledged.

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FURTHER EXPERIMENTS ON THE CARCINOGENICITY OF SYNTHETIC TARS AND THEIR FRACTIONS.

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In a previous communication (*Zeit. f. Krebs.*, 1928, vol. xxvii, p. 308) the results obtained from the application of a pinene synthetic tar to the backs of mice were described, details of the preparation of the tar also being given.

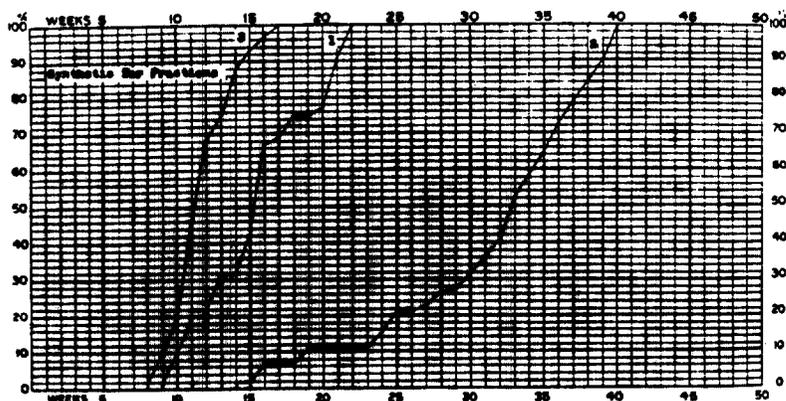
While the method of preparation of the tar remains essentially the same we now employ a much larger electric furnace, the outer tube being wound in four sections and fitted with suitable resistances for temperature control. The internal bore of the inner tube is approximately 75 cm.; it is one metre long. A tube of these dimensions allows us usually to pass a litre of pinene through it without having to remove the carbon from the tube in the middle of the operation—a serious fault in the original furnace with the narrower tube.

It was shown that the active carcinogenic constituents of the tar could be concentrated, and here it is our intention to describe further experiments in this direction. A few remarks on our former experiments will suffice, the general results of which will be intelligible by consulting table I and the accompanying graphs.

Our standard experiment consisted in the application of the agent twice per week between the shoulder blades of 100 mice, the area painted being approximately 5 to 10 mm. in diameter. The graphs are compiled from the percentage of living animals which bore or had borne tumours at any particular week of the experiment. Where a graph of malignant tumours is also given it refers to the percentage of living animals which bore malignant tumours and not to the percentage of tumours which were malignant. The relative potency figures (R.P.) are derived from what we call the potency (P) of each agent, which latter has been calculated from the graph of each experiment compared with the graph of our hypothetical standard agent (H.S.A.) having a potency of 100. The potencies of two gas tars we tested were approximately equal to that of the standard agent. Details of our method for arriving at the carcinogenic potencies of oils and tars form the subject of a paper which is at present in the press, but in order to make our potency figures in this paper intelligible a few remarks on the procedure we have adopted are herewith given.

The H.S.A. graph was compiled from the results obtained in a large

number of experiments with agents of varying degrees of carcinogenic activity and is based upon the skin response of about 30,000 mice. P is arrived at by taking the ratio of the mean percentage number of living tumour-bearing animals per week of the experiment to that of the H.S.A. during a similar period of time, added to the ratio of malignant tumour-bearing animals considered in the same way, multiplied by 100 and divided by 2. The R.P. of an agent is calculated directly, in the majority of cases from the P of the agent compared with the P of some other agent, which latter is presumed to have an R.P. of 100. Thus as P is the percentage potency of an agent compared with the H.S.A., so is R.P. the percentage potency of an agent compared with some other agent X. It must be understood that our figures are only very approximate and while they are a measure of the power of a given agent to induce tumours they do not necessarily give us a true

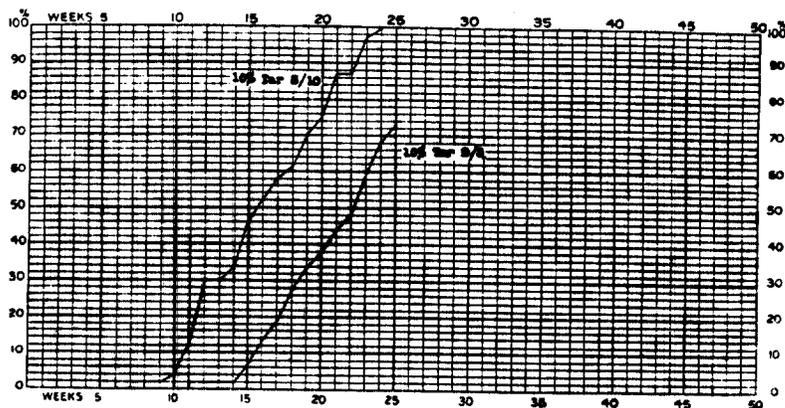


GRAPH 1.

indication of the actual concentration of the carcinogenic material in the agent. This is because there is a definite concentration of the carcinogenic agent to which animals, on the whole, give the maximum relative response.

Considerable concentration of the active principle present in our original synthetic tar (tar B) was effected. Some of the impurities in the nature of low boiling liquid substances were removed on the steam bath, and solids like naphthalene by steam distillation, the residue constituting fraction 1 (about 50 per cent. of the original tar). On removing from the latter the alcohol insoluble substances (fraction 2) we were left with the highly active residue (fraction 3, graph 1). By distillation of fraction 3 up to 200°/3 mm. pressure (fractions 4, 5, 12 and 13) we succeeded in removing a good deal of lowly carcinogenic material. By extracting the potent residue (fractions 6, 7 and 8) with alcohol, an insoluble fraction 9 remained, and on allowing the solution to cool in water, the active principle was precipitated as a solid.

non-active portion precipitating (fraction 11). The final product (fraction 10), which is soluble in cold alcohol, we then endeavoured to concentrate still further. In the pure state fraction 10 did not show itself to be more carcinogenic than fraction 3, but when both fractions were diluted ten times with liquid paraffin the difference in potency



GRAPH 2.

was manifest (see table II and graph 2). The relations of the different fractions to tar B/1 as unity are given below:—

Fraction.	Proportion.	Fraction.	Proportion.	Fraction.	Proportion.
1	1	9	0.025	17	0.028
2	0.578	10	0.099	18	0.076
3	0.427	11	0.049	19	0.054
4	0.101	12	0.056	20	0.023
5	0.152	13	0.197	21	0.026
6	0.113	14	0.015	22	0.050
7	0.055	15	0.037	23	0.024
8	0.008	16	0.043	24	0.030

The percentage concentration in the potent fractions of the active carcinogenic substances from tar B/1 and the percentage loss of active substances in the rejected lowly carcinogenic fractions is shown in the accompanying schema on page 122.

Before proceeding to a further investigation of the carcinogenic activity of fraction 10, the tar was examined as to its constituent elements. It seemed possible that the tar which had been in contact with nitrogen at 850° could have combined to some extent with this element and thereby complicated matters with regard to the carcinogenic substance or substances present, although the qualitative tests for the element were negative. As the result of subsequent treatment it seemed possible also that oxygen compounds might be present. The tar was therefore dried *in vacuo* at 112° for one hour and analysed

for carbon, hydrogen and nitrogen. The latter element was absent and the analytical figures for carbon and hydrogen, for consecutive analyses by micro-methods, were:—C 95.21 and 94.97, H 5.24 and 5.18 which show that the tar is composed entirely of hydrocarbons. The approximate formula for the mixture ($C_{10}H_{12}$) suggests that it is largely composed of condensed rings.

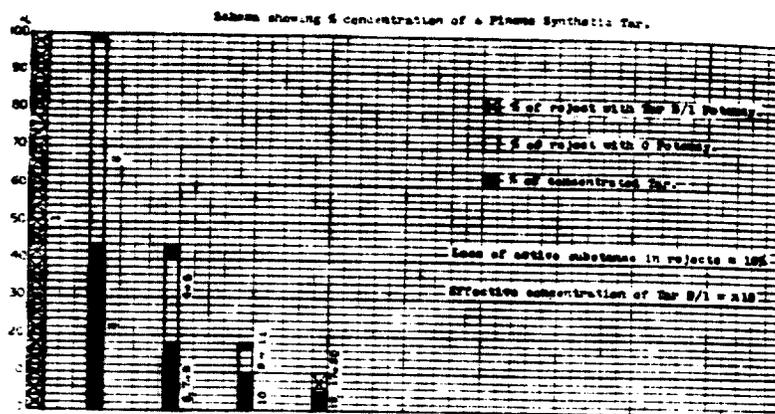


TABLE I.

The carcinogenicity of a pinene synthetic tar.

Tar fractions.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.	R.P.
	Wart.	Epithelioma.	Warts.	Epithelioma.		
Tar B	12th	14th	15	39	25	79
Fraction 1	10th	15th	9	24	25	100
.. 2	15th	34th	7	7	40	4
.. 3	8th	13th	10	10	22	421
.. 4	0	0	40	0
.. 5	16th	25th	12	10	40	10
.. 6	7th	15th	21	17	25	39
.. 7	8th	14th	17	14	25	101
.. 8	12th	20th	7	4	34	21
.. 9	12th	28th	4	4	37	13
.. 10	5th	14th	22	28	22	427
.. 11	15th	31st	4	2	40	7
.. 12	0	0	40	0
.. 13	11th	...	2	0	40	1

R.P. = relative potency compared with that of fraction 1 as 100. The actual carcinogenic potency of fraction 1 was 890.

Three methods were adopted in an endeavour to concentrate the active principle in fraction 10, viz. :—

1. Alcohol extraction.
2. Distillation *in vacuo*.
3. Extraction with alcoholic picric acid.

1. *Alcohol solubility.* Our first procedure was to make a concentrated solution of fraction 10 in hot alcohol, and subsequently to cool in ice. 32 grms. of the tar heated with 800 c.c. of alcohol gave a residue of 1 gm. of a tarry insoluble substance. The solution was cooled in ice when 4.9 grms. of a tarry material separated. This first deposit constituted fraction 14. Half the remaining volume of tar solution was concentrated until turbid, cooled in ice and the deposit obtained (6 grms.) constituted fraction 15. The residue of 7 grms. constituted fraction 16. A 10 per cent. solution of these three fractions was made in liquid paraffin and used for the animal tests. Fractions 15 and 16 were mostly soluble in the liquid paraffin, but in fraction 14 there was a solid sticky portion which did not dissolve.

2. *Distillation.* At the same time we endeavoured to concentrate the active substances in fraction 10 by means of distillation. It was divided into two portions by distilling under a pressure of 5 mm., the dividing temperature being 230° C. The fraction boiling below 230°/5 mm. constituted fraction 17, that above constituting fraction 18. Fraction 17 consisted of crystals together with a small quantity of dark brown oily material. The latter was sucked off at the pump, and the residual crystals constituted fraction 17C. Fraction 18 was darker than fraction 17, and was for the most part crystalline. A 10 per cent. solution of the three fractions in liquid paraffin was used for the animal applications.

It will be seen from table II that we were not equally successful with these two methods of separation of fraction 10. Practically no selective separation of the carcinogenic substances was effected by precipitation from a saturated alcoholic solution, fractions 14 to 16 showing but little difference in activity. All three were somewhat less active than fraction 10 from which they had been derived. On the other hand, distillation effected a moderately clean separation. The fraction boiling below 230°/5 mm. was relatively devoid of carcinogenic activity, while that above was extremely potent. It is true that the latter gave a potency figure less than that of fraction 10, but there are many factors to account for this result as well as for those obtained with fractions 14 to 16. The variation in the animal susceptibility is of course an important factor, and there is possibly a certain degree of variation in the potency of the original batches of tar from which the different fractions have been worked up, as a result of slight variations in the furnace temperature, etc. Also, in the whole of this group of experiments on the concentration of the active principle in our synthetic tar, the loss of a certain amount of the carcinogenic substance may be really only apparent. It is not unlikely that many of the crystalline substances which have been separated out in our relatively non-toxic fractions are in effect highly active when in solution. Some of these crystalline compounds are little soluble in any of the ordinary organic solvents, and we are seeking a non-

carcinogenic solvent which will allow us to test the degree of real activity of the compounds by allowing the substance to come into intimate contact with the animal's skin.

TABLE II.
The carcinogenicity of fractions of a pinene synthetic tar diluted with liquid paraffin.

Tar fractions.	Percentage dilution.	Week of first tumour.		Total number of tumours.		R.P.	Duration of experiment in weeks.
		Warts.	Epithelioma.	Warts.	Epithelioma.		
3	10	11th	22nd	11	5	100	25
6	10	9th	21st	6	4	27	25
10	10	6th	11th	11	37	426	25
13	10	0	0	0	40
14	10	9th	20th	17	11	139	25
15	10	8th	15th	16	13	239	25
16	10	11th	15th	17	35	190	25
17	10	17th	20th	5	6	64	40
18	10	6th	15th	15	16	350	25
19	10	5th	6th	7	20	749	25
20	10	16th	27th	4	3	58	40
23	10	7th	11th	14	24	750	25
24	10	6th	14th	14	27	517	25
25	10	8th	16th	10	33	495	25
26	10	10th	19th	4	1	38	25
21	5	5th	9th	8	7	255	25
22	5	8th	10th	9	4	444	25
8	1	23rd	...	1	0	0.5	40

R. P. is the relative potency compared with that of tar B; 3 10 per cent. as 100. The actual carcinogenic potency of tar B 3 10 per cent. was 176.

3. *Picric acid extraction.* In previous experiments we had found that an effective concentration of the active carcinogenic principle in shale oils could be obtained by treating the oils with a mixture of methyl sulphate and picric acid, or even better by treating with picric acid in alcohol (this *Journal*, 1929, vol. xxxii, p. 149). Consequently we decided to utilise picric acid to obtain, if possible, crystalline picrates of the active constituents of our tar. Fraction 10 was treated as follows. To 30 grms. of the tar in 500 to 600 c.c. of hot absolute alcohol were added 30 grms. of picric acid. On cooling a dark red crystalline mass of mixed picrates separated, which could be recrystallised from the same solvent. After filtering off the deposit the solvent was evaporated on the steam bath to half its bulk and the whole again cooled, but no further separation of picrates took place, even on further concentration. The picrates of the hydrocarbons in benzene were decomposed by means of sodium carbonate solution and the parent substances recovered. Excess picric acid was removed from the residue in the same way, after distillation of the bulk of the alcohol. In this way 12.8 grms. of non-picrate-forming hydrocarbons were obtained and by difference 17.2 grms. of combining material.

A 10 per cent. solution in liquid paraffin of the extract (designated

fraction 25) and of the residue (designated fraction 26) were utilised for the applications in two standard experiments and the results obtained were of considerable interest (table II). The residue was almost devoid of activity while the extract proved to be appreciably more potent than fraction 10, the results of the two experiments considered conjointly indicating a considerable degree of concentration. The relative potencies of the extract and the residue, compared with the original fraction 10 with an R.P. of 100, were 117 and 8 respectively. We next endeavoured to concentrate the picric acid extract (fraction 25) by means of distillation at a pressure of 2 mm. by which means five fractions were obtained with boiling ranges:—below 190°, 190 to 210°, 210 to 240°, 240 to 270° and above 270°. A 1 per cent. solution by weight of the tars in chloroform was used for animal tests, the applications being made five times per week. From the evidence at present available it appears that the bulk of the carcinogenic material is located in the higher boiling fractions.

The concentration of fraction 18. In view of our results with fractions 17 and 18 we decided to re-divide fraction 18 by distillation. It was distilled up to 260°/4 mm., and a 10 per cent. solution of the residue (fraction 19) and of the distillate (fraction 20) in liquid paraffin were tested on animals. A sticky solid, practically insoluble in the paraffin, formed a large part of the residue, while the crystalline distillate was almost entirely insoluble in cold paraffin. Fraction 18 was also separated into two further portions by means of alcohol. A 5 per cent. solution in liquid paraffin of the alcohol insoluble portion (fraction 21) and of the alcohol soluble portion (fraction 22) were used for the applications.

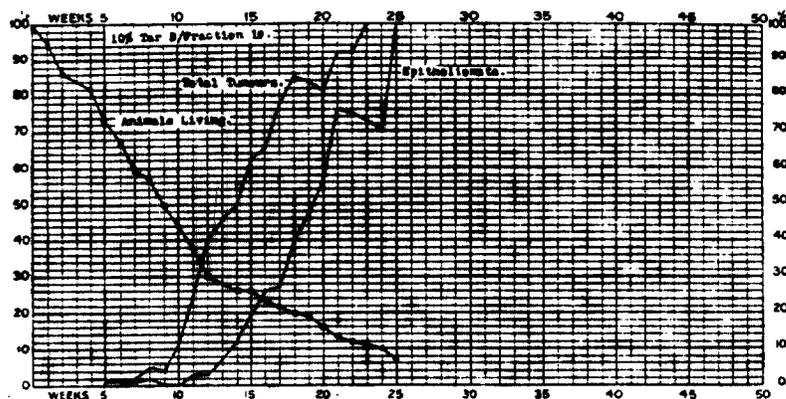
A moderately clean separation of fraction 18 was effected by distillation, the dividing temperature being, as stated, 260°/4 mm. Fraction 20, the lower boiling portion, equivalent to that portion of fraction 10 boiling between 230 to 260°/4 mm., was but little toxic, while fraction 19, the higher boiling portion, was very active.

The first tumour among the animals painted with a 10 per cent. solution of fraction 19 occurred in the fifth week of the experiment, and by the sixth week it had become malignant. This is the earliest experimental malignant tumour which we have encountered among 35,000 animals treated with different carcinogenic agents. It is true that occasionally a tumour may arise among a batch of animals during the first week or so of an experiment, and not uncommonly these precocious tumours rapidly assume malignant features. However, in order not to confuse matters such tumours have been regarded by us as of spontaneous origin (or equivalent to being of spontaneous origin) having due regard to the potency of the agent which is being utilised for the applications.

Graph 3 shows the degree of carcinogenic activity of this fraction of the tar, it being appreciably greater than that of fraction 10, the most concentrated fraction we had previously tested, the potency figures being 175 to 100.

We found, on the other hand, that alcohol was of little value for dividing fraction 18, as there was not sufficient difference in the carcinogenic activity of the two fractions obtained (fractions 21 and 22). Both fractions were however very potent, especially fraction 22 which was stronger even than fraction 19, fraction 21 being somewhat less potent than fraction 19 (table II). These two experiments were also unsatisfactory on account of the high mortality among the animals.

The concentration of fraction 19. It was evident that the separation of fractions 10 and 18 by means of distillation gave far better results as regards the concentration of the active principle of our synthetic tar than did differential solubility in alcohol, the reverse being the case in our earlier experiments when we were separating fraction 3. We thus continued to utilise distillation, and fraction 19 was divided into two further portions by collecting the distillate



GRAPH 3.

which boiled up to $290^{\circ}/4$ mm. (called fraction 23) while the residue in the flask constituted fraction 24. A 10 per cent. solution in liquid paraffin of these two fractions was used for animal tests. Fraction 23 was of a brick red colour and semi-crystalline, fraction 24 having the appearance of pitch. The former was very little soluble in liquid paraffin, the latter moderately so. The difference in potency of these two fractions will be seen in table II, and our results indicate that we had reached the limit of temperature at which the carcinogenic component of fraction 10 could be successfully concentrated by means of distillation, as other results had shown that further concentration could not be effected by differential solubility in alcohol. Thus we resorted to picric acid extraction of fraction 19 as an alternative method, this reagent having proved useful for the separation of the active compounds from fraction 10. Fraction 19, unfortunately, did not lend itself to this procedure so well as fraction 10, as the material was little soluble in alcohol, and while readily soluble in benzene

treatment with picric acid in the latter solvent did not cause any crystalline picrate to separate. Later, crystalline picrates were obtained from the alcohol soluble portion of fraction 19, which we are proceeding to test on animals.

The daily application of diluted tars.

In view of the high potency of some of the later fractions of our tar we decided to test the effect of a 1 per cent. solution instead of a 10 per cent. solution with the object of ascertaining more accurately, if possible, the actual amount of concentration which had been effected. Accordingly a 1 per cent. solution of fractions 10, 19, 23 and 24 in liquid paraffin were tested on mice, the applications being made daily, five times a week, instead of twice a week as in the standard experiment. Details of the results of this group of experiments are given in table III, wherein it will be seen that by adopting the above procedure the

TABLE III.
Daily applications of 1 per cent. solutions in liquid paraffin of synthetic tars.

Tar fraction.	Week of first tumour.		Total number of tumours.		R.P.
	Wart.	Epithelioma.	Wart.	Epitheliomata.	
10	19th	28th	6	7	100
19	13th	18th	13	17	231
23	13th	20th	12	9	252
24	8th	18th	9	17	352

The duration of each experiment was 35 weeks.

R.P. is the relative carcinogenic potency, the 1 per cent. solution of tar B/10 being taken as standard.

figures relating to the active component in the tars were somewhat different to those obtained by utilising the standard experiment. The most powerful fraction, fraction 24, as a 1 per cent. solution had a potency of 250 which indicates that in the pure state it contained about 300 times as many carcinogenic units per c.c. as an ordinary coal gas tar.

Dilution of tars with volatile solvent.

It occurred to us that the utilisation of a large quantity of liquid paraffin, while in itself inert, might offer some protection to the animal from the noxious action of the tar. Numerous other experiments with oils and tars had not shown this to be so, but in any case we imagined that to ascertain the minimal amount of a given substance necessary to induce tumour development, it would be advisable to utilise a volatile solvent, which would leave the substance in the pure state, in very

small quantity, in contact with the skin of the animal. We used the same four fractions of the tar as in the liquid paraffin experiments mentioned above, the tars being first diluted 200 times by weight with chloroform, this substance appearing to be the best solvent for the four fractions. We used the standard method of experiment. The available results are given in table IV, and they more or less confirm the results

TABLE IV.

The carcinogenicity of synthetic tar diluted 200 times with chloroform.

Tar fraction.	Week of first tumour.		Total number of tumours.		R.P.
	Wart.	Epithelioma.	Wart.	Epitheliomata.	
10	11th	...	7	0	100
19	9th	21st	32	1	459
23	7th	21st	19	2	403
24	7th	12th	31	1	524

The duration of each experiment was 25 weeks.

obtained with the same fractions of the tar where other procedures for animal tests have been adopted. The potency figures calculated from the results of the 12 experiments performed with these four fractions of the pinene tar are massed in table V in order to show clearly the relative response of the animals to each tar. The figures relating to the 10 per cent. dilution of fraction 24 would appear to be too low; this may be due to a lack of solubility of the active constituents of the tar, although insusceptibility of the batch of animals used might easily account for the irregularity of our results.

TABLE V.

Potency figures relating to four fractions of a synthetic tar tested under different conditions.

Tar fraction.	10.	19.	23.	24.
10 per cent. in liquid paraffin, R.P.	100	172	183	121
1 " " " (daily) R.P.	100	231	252	352
0.5 " in chloroform R.P.	100	459	403	524
Average R.P.	100	287	279	332

R.P. is the relative potency compared with that of fraction 10 as 100. The carcinogenic potency (P) of tar B/10 tested in different ways was:—

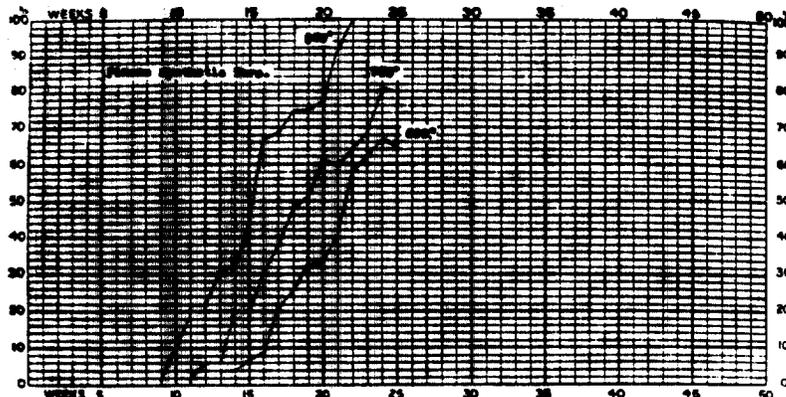
Standard experiment, pure	P.
" " " 10 per cent. in liquid paraffin	3302
" " " 0.5 per cent. in chloroform	750
Daily, 1 per cent. in liquid paraffin	29
	71

Our experiments seem to show that if one wishes to obtain the greatest possible number of tumours from the application of a given number of carcinogenic units then it is in some cases best to dilute to a certain extent, the dilution figure varying with the original strength of the agent under consideration. Similarly, from the point of view of economy of material it is sometimes better to paint the animals every day and in other cases only very infrequently. In order to find the minimal quantity of our tar which is capable of inducing the development of tumours we have instituted further dilution experiments. Animals are being painted twice a week and five times a week with 1 in 1000 and 1 in 10,000 dilutions of fraction 19 in chloroform. So far tumours have been obtained among the animals painted with the 1 in 1000 dilutions even when the applications were only made twice per week. In view of these results one can assume that more frequent applications of a 1 in 10,000 dilution will almost certainly give rise to tumours at a later date.

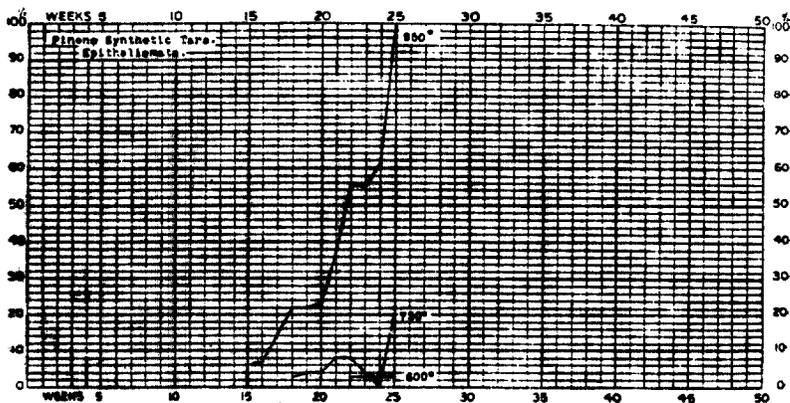
The potency of tars made at different temperatures.

Our original synthetic tar which we had found to be very active was made from pinene by passing it through a vitreous tube in an atmosphere of nitrogen at a temperature of 850° to 870° C. It seemed to us of interest to investigate what the effect of varying the temperature of the tube would be, Keenaway (*Brit. Med. Journ.*, 1925, ii. 1) having already shown that the temperature of manufacture of a synthetic tar was an important factor in the yield of carcinogenic substance. As the amount of carbonisation taking place in the furnace tube varies greatly with the temperature, we hoped to facilitate the manufacture of the tar by using a temperature lower than 850° C. While we found that little or no carbonisation took place at the lower temperatures, these low temperature tars proved to be less carcinogenic than our original tar made at 850° to 870° C. Three tars were prepared, at 500°, 600° and 750° C. and they were tested on animals in the usual manner. It was found that the yield of active material decreased as the temperature of manufacture was lowered, this being clearly shown in graphs 4 and 5 and table VI. The activity of fraction 1 of tar B/500 was such that we have had up to the present only one very doubtful benign tumour which healed and left a practically normal epithelium, so that this tar is meanwhile registered as having an almost negligible potency. In a further experiment we found that pure untreated pinene was not above suspicion, one of two animals surviving at the present time having recently developed a papilloma (55th week). On the contrary a polymerised pinene, a sticky resinous material, induced no tumour formation in a standard experiment of 78 weeks' duration.

A higher temperature tar was prepared from pinene by heating the furnace tube to 950° C. The yield of tar was very small owing to carbonisation, and several runs had to be made to obtain sufficient for



GRAPH 4.



GRAPH 5.

TABLE VI.

The effect of varying the temperature of the furnace tube.

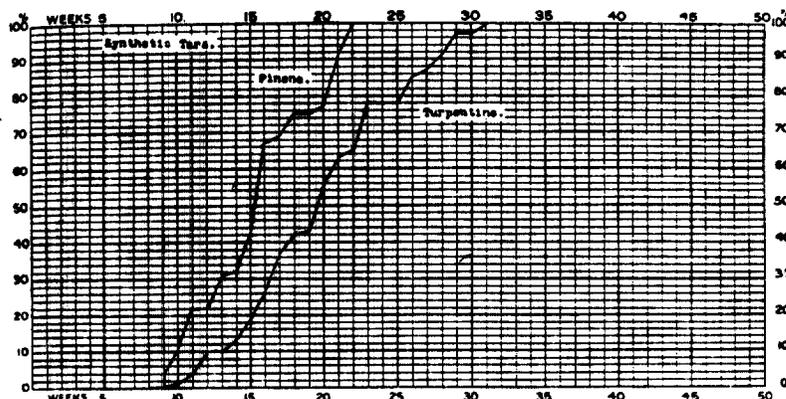
Tar.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.	R.P.
	Wart.	Epithelioma.	Warts.	Epitheliomata.		
Tar B/1/500°	12th	...	21	0	40	10.1
" B/1/800°	13th	22nd	17	16	40	12
" B/1/750°	11th	18th	14	7	25	24
" B/1/850°	10th	18th	9	24	25	100
" B/1/950°	7th	16th	24	38	25	64
" D/1/850°	8th	19th	25	36	40	21

Tar B/1—Fraction 1 of a pinene synthetic tar.

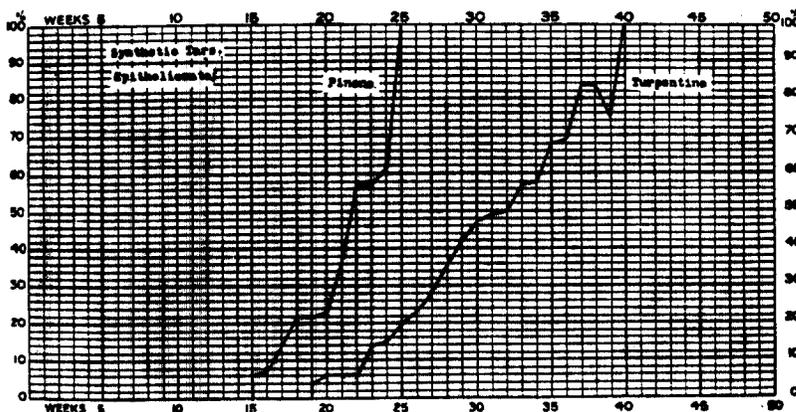
Tar D/1—Fraction 1 of a turpentine synthetic tar.

R. P.—Relative potency compared with that of tar B/1/850° as 100.

a standard experiment, but the amount of carcinogenic material present in the tar was large, although apparently a good deal less than in the tar made at 850° C. (table VI). This group of experiments is of interest in connection with the exposure of food-stuffs (meat, toast, etc.) to the temperature of the kitchen oven, and the possible formation of hydrocarbons. If the oven temperature happens to be high enough for the



GRAPH 6.



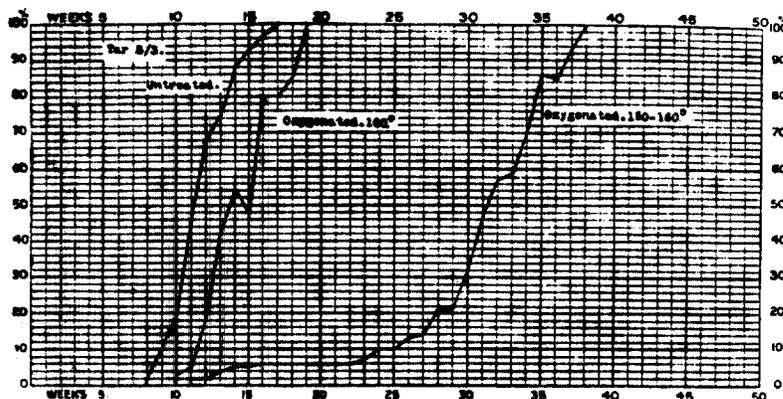
GRAPH 7.

synthesis of carcinogenic compounds, the latter, however small in quantity, may have a bearing on the aetiology of alimentary cancers.

Turpentine tar.

With a view to lowering the cost of production of our tar we tested the efficacy of a turpentine tar. A pure american turpentine, distilled from the resin, was passed through the vitreosil tube at 850° C. and the resulting tar residue, after steam distillation, was tested on mice. It will be seen from graphs 6 and 7 that the activity of this tar is

appreciably less than that of the pinene tar made under similar conditions, and corresponds more nearly to that of the pinene tar made at 750° C. (see table VI). These results are in accordance with those of Kennaway who found that the yield of active material in tars prepared from two different simple hydrocarbons was not necessarily equal in amount. We decided therefore that it would be unwise to substitute turpentine for pinene for the regular manufacture of our tars. Here again the material we were utilising for the manufacture of our tars was tested on animals before use. The turpentine failed to induce the development of tumours, with the exception of a doubtful small papilloma, but induced a hyperplasia of the epithelium with moderate regularity.



GRAPH 8.

The oxidation of synthetic tars.

Oxygen was bubbled through fraction 3 of a pinene synthetic tar for 24 hours, the tar being heated on a steam bath. A second experiment was performed in a similar manner with the temperature of the bath raised to 150° to 160° C. Oxidation at 100° did not materially alter the physical properties of the tar, but at the higher temperature made the tar blacker and much more viscous. The carcinogenicity of the tar had been only slightly affected by oxidation at 100° but profoundly at 150° to 160°, the percentage decreases in potency being 27 and 99 respectively. Graph 8 shows clearly the diminished activity of the two oxygenated tars as compared with the control untreated tar. It is possible that at a still higher temperature treatment with oxygen would have rendered the tar inert. However, it must be understood that the tar oxygenated at 150° to 160° was only relatively inactive for it retained a potency of 58, which figure is approximately that of a shale oil or somewhat less than that of ordinary gas tars. Of the animals painted with the tar oxygenated at 150°, 15 developed malignant tumours; in several cases there were metastases in the lungs and the neighbouring lymph glands.

which is accounted for by the fact that the duration of the experiment was longer than that usually employed by us for the testing of active synthetic tars.

One animal of this series developed a *sarcoma* at the site of the tar applications. During the 14th week of the experiment a wart was observed which some time later fell off, leaving an ulcerated surface on the skin. A small hard lump was palpable by the 28th week, which was recorded as a clinical epithelioma. Unfortunately the animal died during the following week, and the tumour, on section, was found to be a round celled sarcoma, the first tumour of this nature we have observed among approximately a thousand experimentally induced malignant skin tumours.

We next oxidised the tar by permanganate. To 30 grms. of fraction 3 of the pinene synthetic tar in 300 c.c. of pure acetone at the ordinary temperature were added 20 grms. of finely ground potassium permanganate in small quantities. There was a considerable evolution of heat, the temperature rising to 40° to 45° C. The precipitated manganese dioxide was removed by filtration, and to the filtered solution was added a further small quantity of permanganate but decolorisation was slow and no rise in temperature was noted. A little alcohol was added to remove unchanged permanganate, and after filtration the solvent was removed by distillation. The tar was then taken up in ether and washed with sodium carbonate solution, followed by water. After drying over calcium chloride the solvent was removed on the water bath, and finally *in vacuo*. A yellow acid was obtained from the manganese dioxide precipitate on extracting with water and acidifying the concentrated liquors. It appears to be an anthracene carboxylic acid, arising no doubt from a conversion of an alkyl derivative of anthracene to a carboxylic acid derivative by means of permanganate. Its m.p. from benzene is 261° to 263°.

A 10 per cent. solution of the permanganate-treated tar was used for the applications, but the experiment was somewhat spoiled as the batch of animals concerned did not live well. However, it is evident that the permanganate had an appreciable influence on the carcinogenic components of the tar for the number of tumours obtained was small and none of them became malignant, although the duration of the experiment surpassed that of the control. A tendency for the papillomata which developed to heal was also noticeable, a feature frequently associated with weak agents of a certain category. The relative potency of the acetone-permanganate treated tar was 24, being thus 76 per cent. under par.

The same tar, in pyridine solution, was also oxidised by means of permanganate, the amount of oxidising agent employed being varied in two experiments.

To 30 grms. of tar B/3 in solution in 150 grms. of water and 450 c.c. of pyridine on the steam bath were added 50 grms. of finely ground permanganate in 20, 20 and 10 gm. lots, with vigorous shaking. Much heat was evolved. After two hours on the water bath the precipitated manganese dioxide was

filtered off and washed with 100 c.c. of pyridine. A further 100 c.c. of water were added, followed by 50 c.c. of pyridine so that the tar remained in solution. A further 50 grms. of permanganate were added as before and the solvent, after filtration of the manganese dioxide, was removed *in vacuo*. The product was a sticky tar, but little soluble in benzene and containing much acidic material. On shaking with 200 c.c. of benzene and dilute sodium hydroxide, to take up the acids present, a small amount of insoluble material (0.4 grms.) remained, and proved by melting-point and mixed melting-point with a pure specimen (when no depression was noted) to be anthraquinone. The alkaline layer was saturated with carbon dioxide but no precipitation resulted. Acidification with hydrochloric acid gave a solid body (this was retained). After washing the benzene layer with dilute hydrochloric acid to remove traces of pyridine, the solution was dried over calcium chloride, and the semi-crystalline solid (15 grms.) remaining on removal of the solvent finally *in vacuo* was used in 10 per cent. liquid paraffin solution for the animal applications. The manganese dioxide on extraction with hot water followed by acidification yielded no acid product.

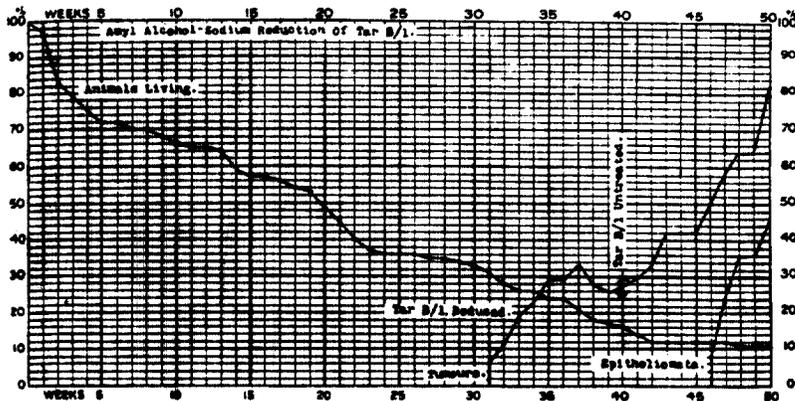
In the second experiment 30 grms. of the same tar fraction in 450 c.c. of pyridine and 100 c.c. of water heated on the water bath were mixed with 40 grms. of finely ground permanganate added in small quantities over four hours. After filtration of the manganese dioxide the solvent was removed and the product extracted with sodium hydroxide and benzene as before. A non-crystalline insoluble residue (2 to 3 grms.) remained. The benzene solution treated as above yielded 21.2 grms. of substance which was used in 10 per cent. liquid paraffin for the animal test. The acid material from acidification of the alkaline solution was unpromising in nature, and was discarded.

The animal experiments showed that while 100 grms. of permanganate renders 30 grms. of tar B/3 almost inert, 40 grms. of permanganate is not sufficient, the oxidised tar still having a relative potency of 17 per cent. of that of the original tar.

The reduction of the synthetic tar.

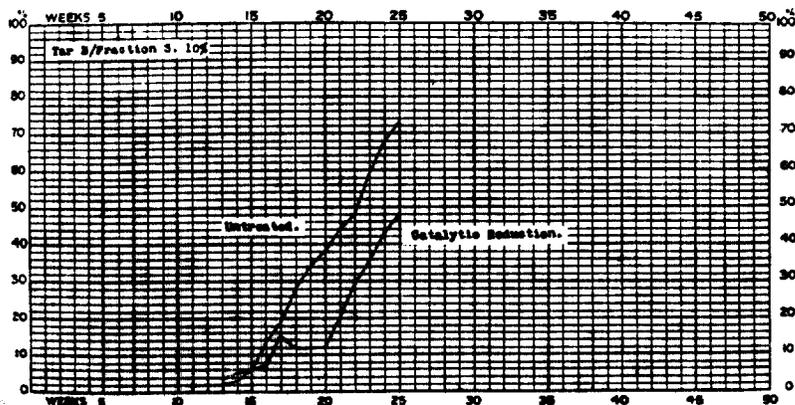
In the first experiment of this nature the highly carcinogenic fraction 1 of our pinene synthetic tar was reduced by the action of sodium on boiling amyl alcohol. After the reaction the amyl alcohol was removed by steam distillation. The reduced tar was similar in appearance to that of fraction 1 before treatment. The results of this experiment showed that we had practically detoxicated the tar as far as its carcinogenicity is concerned, the first tumour not appearing until 31 weeks after the commencement of the applications. In the control animals painted with untreated fraction 1 the earliest tumour appeared in the 10th week, while by the 22nd week all the animals of the series remaining alive were bearing tumours, the majority of which were already malignant. From table VII we see that the reduced tar failed to induce the development of a malignant tumour when the painting was continued for 40 weeks. At this time, when only 16 animals remained, the applications of the reduced tar were discontinued, untreated fraction 1 being substituted for it. Malignancy then rapidly supervened, the extent of which will be appreciated by consulting

graph 9. The amyl alcohol sodium reduction was apparently as powerful a method for detoxicating a tar as oxidation at 150° to 160°, the relative potency figures being very low.



GRAPH 9.

We next attempted catalytic reduction by means of palladium, and in this our efforts were not so successful, a tar so treated losing only 15 per cent. of its original toxicity (graph 10 and table VII). For the animal test the tar was diluted ten times with liquid paraffin, and applied in the ordinary manner. This experiment we are repeating



GRAPH 10.

as the amount of hydrogen absorbed was comparatively small. The procedure was as follows.

The catalyst was prepared by dissolving 1 gm. of palladium chloride in 100 c.c. of water with the addition of two drops of concentrated hydrochloric acid. Solution occurs after several days, and 12 grms. of animal charcoal ("acetite") previously heated to redness were then added. The catalyst was then

reduced in an atmosphere of hydrogen, purified by passing through solutions of silver nitrate, permanganate and sodium hydroxide. When hydrogen ceased to be absorbed 19 grms. of the tar were added in acetic-ethyl acetate solution through a tap, and hydrogen under slight pressure was slowly absorbed during vigorous mechanical stirring of the mixture. Absorption ceased at the end of 40 hours when 750 c.c. of hydrogen had been taken up. If the molecular weight of the substances in solution lay between 200 and 300 it would appear that only a small amount of material had been affected. Addition of hydrogen to a double bond would occur most readily, while the possibility of hydrogenating a nucleus such as phenanthrene in the 9 to 10 position is also feasible. The catalyst was filtered off on a Buchner funnel and washed with ethyl acetate. It can be used again after exposure to sun and air for some hours. The product on removal of the solvent was employed diluted for the animal test. In a second experiment using ethyl acetate as solvent for the tar only 135 c.c. of hydrogen were absorbed by 13.5 grms. of material. A distinct smell of pyridine was noted on heating the charcoal to redness and this substance may have acted as a catalyst poison.

TABLE VII.
Oxidation and reduction of pine synthetic tars.

Material.	Week of first tumour.		Total number of tumours.		Warts Epitheliomas	R.P.
	Warts.	Epithelioma.	Warts.	Epithelioma.		
Tar B 1	10th	13th	9	24	25	100
.. reduced amyl alcohol	31st	..	8	0	40	2.5
.. reduced amyl alcohol de- hydrogenated with sulphur	14th	26th	13	2	37	5
Tar B 3	8th	13th	10	40	22	100
.. oxygenated at 100° C.	10th	14th	17	6	22	68
.. .. 150-160° C.	10th	13th	12	15	40	1
.. 1/10 liquid paraffin	11th	22nd	11	5	25	100
.. sulphur treated	12th	18th	18	7	25	80
.. acetone-permanganate	12th	..	5	0	28	24
.. pyridine - permanganate 40 grms.	16th	22nd	4	2	34	17
.. pyridine - permanganate 100 grms.	0	0	34	0
.. catalytic reduction	13th	21st	13	1	25	85

R.P. = relative carcinogenic potency.

The actual potency of tar B 1 = 590.

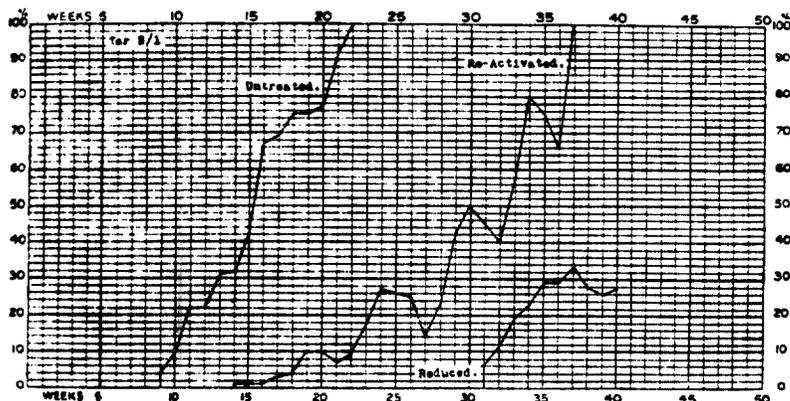
.. B 3 = 3745.

.. of 10 per cent. tar B/3 = 176.

It remained now to see whether we could re-activate our reduced tars by removing the hydrogen which we had added by reduction methods to the material in the original tar fraction. Our first procedure was to treat our reduced fraction 1 in an oil bath at a temperature of 140° C. with sulphur, an agent often employed for dehydrogenation in organic chemistry. Six grms. of the reduced tar were heated with 6 grms. of sulphur for some hours till hydrogen sulphide ceased to be evolved. A further 0.6 grms. of sulphur were added, and the heating continued for 24 hours. The product was extracted with benzene and the solution filtered from suspended

material. Table VII and graph 11 show that we had increased the activity of the reduced tar ten-fold, but that the carcinogenic potency was still only 5 per cent. of that of the original tar before reduction.

We also endeavoured, by a similar procedure, to render our active fraction 3 of the synthetic tar still more active, 38 grms. of the tar being heated with 6.3 grms. of sulphur for 30 hours at 140°. A further 6 grms. of sulphur were added and heating continued for another 12 hours, the temperature of the oil bath being gradually raised to 160° to 170°. The product was extracted with benzene, and a considerable amount of carbonisation had taken place. It will be seen from the table that this experiment was unsuccessful, the sulphur treatment having deprived the tar of 20 per cent. of its original activity. It is possible that while activation of previously inactive



GRAPH 11.

compounds present in the tar resulted, the nature of the reaction was such that many of the active compounds already present were rendered inert. Meanwhile in some experiments we are substituting bromine for sulphur.

Silica gel adsorption.

Our results on the oxidation, reduction and re-activation of tars have in the main confirmed similar experiments performed on oils (this *Journal*, 1929, vol. xxxii. p. 149) and in view of the partial detoxication of shale oil by means of percolation through a long column of silica gel we attempted to detoxicate our synthetic tar in the same way. It was necessary to have a hot jacket round the percolation tube to reduce the viscosity of the tar. A 10 per cent. solution in liquid paraffin of the percolated tar and the residue held up by the gel were used for the animal tests, but the experiments were of little use because of the heavy death-rate among the animals. As far as one could judge tars so treated lost a slight amount of their carcinogenic activity.

The carcinogenicity of some pure hydrocarbons.

Experiments in this direction have already been performed by several workers, but as far as we are aware no success has been met with in the production of tumours by these means. We decided to experiment along these lines, not only with the object of tumour production but also to get, if possible, an indication of the nature of the active component of the tars by the hyperplastic response of the epithelium. The significance of the hyperplasia we have discussed elsewhere (*Journ. of Hygiene*, 1928, vol. xxviii. p. 219).

We prepared two mixtures in the first place.

Mixture 1. This consisted of anthracene, anthracene dihydride, β -methyl anthracene, phenanthrene, retene, fluorene and acenaphthene. Two grms. of each were dissolved in 100 c.c. of chloroform. Anthracene hydride and β -methyl anthracene we prepared synthetically by the usual methods. Fluorene and acenaphthene were kindly supplied to us by Messrs Harden and Holden of Manchester.

Mixture 2. This consists of a suspension of chrysene, truxene and benzerythene; 1.3 grms. of each was dissolved in 50 c.c. of hot liquid paraffin and the solution cooled so that the crystals should be as small as possible. Truxene and benzerythene were prepared synthetically by the usual methods (*Annalen der Chemie*, 1904, cccxxxii. 52, *Journ. Chem. Soc.*, 1894, lxxv. 276).

The experiment with mixture 1 was continued over a period of 70 weeks, but we failed to observe any tumour formation and the response of the epithelium to the applications was negligible. It is tolerably certain that the part played by the compounds present in this mixture in the induction of tar cancers is practically *nil* although the possibility of an increase in their activity when dissolved in other constituents of the tar cannot be ignored.

The experiment with mixture 2 was more successful as two rapidly growing epitheliomata were observed among the animals painted with this mixture for 60 weeks (see table VIII). In a second experiment the death-rate of the animals was so high that there was little chance for malignant tumours to develop. The five animals which were killed at the 60th week had skins which showed a well-marked hyperplasia of the epithelium and subepithelial inflammation. Altogether the sections were microscopically similar to those obtained from animals painted for a short while with ordinary gas tar.

These results we considered of sufficient importance to warrant further experiments. We proceeded first to test each of the three substances separately, together with the liquid paraffin in which they were emulsified. Also, as the chrysene we used in our mixture 2 experiment was not pure, we re-crystallised this substance twice, and utilised the pure material and the residue for animal tests. In three experiments we substituted oleic acid for the liquid paraffin to over-

come any protective action of the latter and at the same time possibly to aid the active substance. To make the experiments as nearly as possible comparable with those performed with mixture 2, a 7.5 per cent. suspension of each substance in the diluents was used. Oleic acid proved to be a poor solvent especially for chrysene, this substance being very insoluble in all organic solvents and forming a thick paste when diluted with liquid paraffin.

TABLE VIII.
The carcinogenicity of some pure hydrocarbons and mixtures.

Hydrocarbon, etc.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.
	Wart.	Epithelioma.	Warts.	Epitheliomata.	
Mixture 1 in liquid paraffin	0	0	0	70
Mixture 2 " " " . . .	46th	45th	1	2	60
" 2 (second test) . . .	44th	...	1	0	60
Chrysene pure in liquid paraffin .	19th	65th	1	1	70
" impure " "	0	0	30
Truxene in liquid paraffin "	0	0	50
Benzerythrene in liquid paraffin .	? 30th	...	? 2	0	40
Chrysene residue " " . . .	33rd	...	2	0	50
" impure in oleic acid . . .	9th	50th	5	2	85
Truxene in oleic acid	0	0	50
Benzerythrene in oleic acid . . .	12th	...	1	0	50
Dodecahydrotriphenylene	0	0	50
Dodecahydrotriphenylene sulphur treated	0	0	20

Unfortunately the mortality among the 800 animals utilised for these experiments was high, so that very indefinite results were obtained. More tumours were obtained when using oleic acid as diluent than when using liquid paraffin. Pure oleic acid, as we shall see later, is capable of inducing with regularity an epithelial hyperplasia after prolonged application, and very occasionally it may give rise to a benign papilloma. The meagre results we have obtained will be found in table VIII, and it will be seen that suspicion points towards chrysene as being the most active compound. In six experiments in which chrysene was present in the agent applied we obtained 10 warts and 5 epitheliomata. When chrysene was absent we obtained only one definite tumour in six control experiments, a solitary tumour occurring on a skin which otherwise looked normal microscopically, in an animal painted with an emulsion of benzerythene in oleic acid. Thus benzerythene appears to be capable of inducing tumours, but truxene appears to be inert, even in the presence of oleic acid. Apart from the high death-rate, it is probable that our results would have been more interesting if we had been able to find a suitable solvent. It seems indeed rather remarkable that tumour formation should take place at all as a result of the application of our very unsatisfactory suspensions.

Another unsaturated hydrocarbon, dodecahydrotriphenylene was

selected for animal test. This was prepared by adding 100 grms. of cyclohexanone, purified by means of the bisulphite compound, to 250 grms. of methyl alcohol and 100 grms. of concentrated sulphuric acid, and refluxing for 10 to 12 hours over a free flame, followed by recrystallisation of the solid product from benzol. (*Ber. Deutsch. Chem. Gesellsch.* 1907, xl. 154). As it contains 12 hydrogen atoms more than triphenylene these were considered suitable hydrocarbons for experiment in view of our results on the addition of hydrogen to aromatic bodies. For if we had found triphenylene to be carcinogenic while the reduced body was not, it seemed to us a definite advance would have been made. Triphenylene itself, however, could not be obtained by the method described, and we used sulphur as the dehydrogenating agent. Dodecahydrotriphenylene was heated at 190° to 200° with sulphur in small excess over what theory required to remove the hydrogen atoms, till hydrogen sulphide ceased to be given off. Sodium was added to remove the excess sulphur, and the hydrocarbon distilled *in vacuo*. An attempt to add on six more hydrogen atoms to dodecahydrotriphenylene by means of sodium and boiling amyl alcohol was not successful. The pure dodecahydrotriphenylene and the substance after treatment with sulphur were suspended in liquid paraffin and tested on animals. No tumours have so far been obtained, and on the whole the prospect of tumour formation appears to be remote especially in the case of the pure compound.

In view of the fact that we have found very weak carcinogenic mineral oils to give a much greater yield of tumours when applied daily than when applied only twice a week, we have tested liquid paraffin suspensions of chrysene, benzerythene and pyrene on animals by the more intensive method. This group of experiments has been in progress for 35 weeks during which time each animal has received 175 applications, but without the advent of a single tumour. Such results indicate that the carcinogenic potency even of the chrysene suspension (which is presumably the most active) must be very low indeed, for a weak petroleum oil would have given us a dozen or so of tumours under similar experimental conditions.

Although the potency of our chrysene suspension is so low it is well to remember that this substance is present in large quantities in many of our highly active synthetic tar fractions, and in the presence of other substances is rendered much more soluble. We have isolated chrysene in the pure state from fractions 9, 17 and 20 and in larger quantities from fraction 10. Subsequently attempts will be made to free some of the fractions from chrysene as far as possible, and the residues will be tested for any increase or decrease in carcinogenic potency. Such experiments should tell us more definitely whether this substance is acting as a diluent or as an active carcinogenic agent, the evidence at present available pointing to

the former. The addition of chrysene to some of our low potency tar fractions would have been the easiest way of approaching the subject but unfortunately such experiments were not feasible owing to a lack of solubility of the chrysene in the tars.

The carcinogenicity of fatty acids.

The part played by acids, both organic and inorganic, in the genesis of cancer in our opinion requires serious consideration. It is not known what part hydrochloric acid plays in cancer of the stomach, butyric acid in cancer of the breast, lactic acid in cancer of the breast or sarcomata, or autolytic acids in cancer of the scrotum, etc. Even uric acid may have an effect in the development of papilloma or aniline-dye cancer of the bladder and many other examples might be given of the possible action of acids.

Experimentally the production of cancer by the administration of acids has been on the whole unsuccessful, although it has been claimed that epithelioma of the skin can be induced by the frequent application of very weak solutions of hydrochloric acid. These last experiments have not, to our knowledge, been confirmed.

We have attempted to produce epitheliomata in mice by the continued application of oleic, lactic and butyric acids but have so far not met with any success. The applications were performed twice a week, but as we have had greater success with weak carcinogenic agents when applied more frequently we are now conducting experiments with the acids wherein the applications are made five times per week. By bi-weekly application we have performed three experiments with oleic acid (Kahlbaum) (a) on 100 animals in an experiment lasting 40 weeks which gave no tumours, (b) on 200 animals in an experiment lasting 40 weeks which gave one benign tumour on the 35th week, and (c) on 100 animals in an experiment lasting 23 weeks which gave one benign tumour on the 15th week. Although tumours are rare it is of interest that an unsaturated fatty acid such as oleic acid which is widely distributed in the animal and vegetable kingdom should be capable of inducing tumour formation. Most of the oleic acid we utilised contained about 6 per cent. of saturated acids as impurities, chiefly stearic and palmitic. With lactic acid we failed to induce a tumour in an experiment lasting 25 weeks, and it is probable that if the applications had been continued tumours would not have developed, for the epithelium of the animals examined showed very little indication of hyperplasia. Pure normal butyric acid caused necrosis of the epithelium so that it was applied as a 25 and 50 per cent. solution in olive oil. Some hyperplasia of the epithelium was observed in the early days of the experiments but no tumours developed among the small number of animals used for the test and the general tendency seemed for the early hyperplasia to recede and leave a very frail epithelium. Oleic acid when applied five times per week induced the

formation of a tumour by the 7th week, but as the experiment progresses only an occasional tumour arises, and so far there is no evidence of any change to malignancy in the tumours under observation. However, well-marked hyperplasia of the epithelium of animals which have died is consistently present and we await the advent of malignant tumours with confidence.

In view of the fact that oleic acid is such a consistent hyperplastic agent, experiments were performed with some carcinogenic agents to which a little of the acid was added. It was thought that by this means the activity of the agents might be increased, the experimental results did not, however, confirm our anticipations. When 10 per cent. oleic acid was mixed with a gas tar the mixture had only 64 per cent. of the carcinogenic activity of the original tar. Similarly if oleic acid was used instead of liquid paraffin to dilute our synthetic tar fractions, a lower degree of carcinogenic activity was observed, the results of four experiments of this nature being shown in table IX. Also when 10

TABLE IX.

The effect of the addition of oleic acid to tars.

Material.	Week of first tumour.		Total number of tumours.		Duration in weeks.	R.P.	P.
	Warts.	Epi- thelioma.	Warts.	Epi- thelioma.			
Gas tar 400-450° C.	10th	23rd	8	10	35	100	109
" + oleic acid	9th	23rd	14	9	35	68	74
Tar B/3 10 per cent. Liquid paraffin	11th	22nd	11	5	25	100	176
Tar B/3 + oleic acid	12th	22nd	20	11	25	89	157
Tar B/10 10 per cent. Liquid paraffin	6th	11th	11	37	25	100	750
Tar B/10 + oleic acid	10th	14th	29	43	25	66	492

R.P. = Relative carcinogenic potency.

P. = Standard carcinogenic potency.

and 20 per cent. of the acid was mixed with shale oil, two animal experiments indicated that the activity of the oil was lessened. On the other hand, if 5 or 10 per cent. of shale oil was mixed with the acid the activity of the acid was obviously increased, and malignant tumours were at times obtained. An endeavour is now being made to increase the activity of our tars by dissolving the agent in 1 per cent. of alcoholic potash, in view of the solvent action of alkalis on keratin.

CONCLUSIONS.

1. The carcinogenic activity of synthetic tars varies according to the chemical compound utilised for their manufacture. A synthetic tar made from pinene at 850° C. was a more powerful carcinogenic agent than one made from turpentine under similar conditions. The relative carcinogenic potencies were 100 and 21 respectively.

2. The carcinogenic activity of a synthetic tar varies according to the temperature of the combustion tube. Pinene tars made at 500°, 600°, 750°, 850° and 950° C. had relative potencies of 0, 12, 24, 100 and 64.

3. A synthetic tar was concentrated as far as its carcinogenic activity was concerned by means of distillation and differential solubility in alcohol. A 5 per cent. solution of our most concentrated fraction was about five times as powerful as the ordinary crude gas tars tested by us.

4. The active constituents in the synthetic tar formed crystalline picrates, the residual filtrate being almost devoid of carcinogenic activity. The picric acid extract proved to be extremely potent as a cancer-producing agent.

5. Oxygenation of a synthetic tar at 100° C. reduced the potency of the tar from 100 to 63, and when the same tar was oxygenated at 150° to 160° C. the potency fell to 1. Oxidation with an acetone solution of permanganate reduced the potency to 24; with pyridine as a solvent the figure fell in one case almost to 0.

6. Reduction of the tar by means of sodium in boiling amyl alcohol reduced the potency of the tar to 0.5, and when this tar was treated with sulphur its potency was raised to 5.

7. Dilution of the tar with oleic acid instead of with the inert liquid paraffin reduced the potency by about 20 per cent. Similarly oleic acid reduced the potency of coal gas tar from 90 to 66.

8. Applications of oleic acid have given rise to benign tumours. With lactic or butyric acid no tumours were observed.

9. A mixture of hydrocarbons soluble in chloroform failed to induce the development of tumours while a mixture of insoluble hydrocarbons, suspended in liquid paraffin, gave rise to two epitheliomata.

10. Among several insoluble hydrocarbons tested in the pure state, suspended in liquid paraffin or oleic acid, chrysene (if our specimens have been completely purified) appears definitely to be carcinogenic although its potency is extremely low. Among liquid hydrocarbons tested american turpentine and pinene have both induced the development of a benign tumour.

We would like to acknowledge here that our thanks are due to Mr James Cox for the preparation of many thousand admirable microscopical specimens and to Mr Robert Combes for the care taken in drawing and photographing the graphs.

HEPATIC CHANGES FOLLOWING INTRASPLENIC INJECTION OF FATTY SUBSTANCES

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THERE have been numerous attempts to demonstrate a causal relationship between an increase of fat and an increase of fibrous tissue in hepatic cirrhosis. To date there has been no clear demonstration that these are causally related, beyond the observation that certain fats, such as lard and cod liver oil, enhance the development of experimental fibrosis.¹

The experiments reported here were initiated to study this problem. Fatty substances were injected into the spleen and the evolution of the hepatic changes was followed over a period of months. With this method it was hoped that an agent capable of causing injury to the hepatic mesenchyma would be delivered to the liver over a period of time sufficiently prolonged to induce fibrosis or cirrhosis.

MATERIAL AND METHODS

The following substances were injected intrasplenically:

1. Cod liver oil
 - (a) Cod liver oil.
 - (b) Cod liver oil diluted to 50% in gum acacia as given by Hagerty.²
 - (c) Cod liver oil "neutralized" with calcium hydroxide.
2. Unsaturated fatty acids
 - (a) Oleic acid, diluted to 50% in gum acacia.
 - (b) Linoleic acid, diluted to 50% and to 25% in gum acacia.
3. Glycerol and liquid petrolatum
4. Rat tissue emulsions
 - (a) Emulsion of rat body fat, diluted to 50% in gum acacia. Three grams of retro-peritoneal fat of rats was emulsified with 0.75 to 1.0 gm. of powdered acacia and diluted with 27 cc. of distilled water.²
 - (b) Emulsion of normal liver of rat. This was prepared as described above.
 - (c) Emulsion of fatty liver of rat, prepared as described above. The fatty livers were obtained following a high-fat, low-protein diet administered for a period of 8 to 10 weeks.

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1. Györgi, P.: Liver Injury, in Research in Medical Science, edited by D. E. Green and W. E. Knox, New York, The Macmillan Company, 1950.

2. Hagerty, C. S.: Experimental Embolic Glomerulonephritis Produced with Human Fat, Fatty Acids and Calcium Soaps, Arch. Path. 25:24 (Jan.) 1938.

with small amounts, 100-150 μm, of a single strain, were used in all experiments.
The animal was anesthetized with ether, the spleen was exteriorized, and the material to be tested was injected directly into the spleen. Often there was slight spilling of the fatty material into the peritoneal cavity from the site of splenic injection. Only one injection was administered. The amounts ranged from 0.15 to 0.5 ml. in the different animals.

The animals were killed at regular intervals as shown in the accompanying table, by etherization and exsanguination.

A few rats were splenectomized 24 hours after intrasplenic injection of the tested material and later killed as noted in the table.

Pieces of right and left lobes of the liver and of the spleen were put into Baker's calcium-cadmium formalin³ and either Zenker's acetic acid solution or Tellyesniczky's fluid. Frozen sections were cut from the formalin-fixed blocks at 10 μ and stained with the following stains: Sudan IV; a combination of Laidlaw's silver method and Sudan IV; Nile blue sulfate; toluidine blue. Paraffin sections were stained with hematoxylin and eosin, Heidenhain's azocarmine modification of Mallory's connective tissue stain, and Weigert's hematoxylin-Van Gieson stain.



Fig. 1.—Liver five minutes after intrasplenic injection of cod liver oil. Interlobular twigs of the portal vein are distended by oil droplets. $\times 350$. (This and the following pictures were taken from hematoxylin-and-eosin preparations except where otherwise stated.)

OBSERVATIONS

Cod Liver Oil.—Within five minutes after injection, fat droplets were lodged within the portal vascular tree, where they remained for several weeks and disappeared within a month. For the most part the oil lodged in the smaller branches of the portal system, beyond the spaces of Glisson's capsule in vessels which lay between liver lobules (Figs. 1 and 2A). Occasionally a small portal branch in Glisson's capsule was occluded by the fatty substance. The greatest number of oil droplets was found in portal vessels beneath the capsule, near the free margin of the lobes. All the changes which occurred took place in relation to the occluding fat droplets. No oil escaped into the sinusoids or the systemic vessels of the liver.

During the first five hours there were accumulations, or small clusters, of polymorphonuclear neutrophiles along the endothelium of the obstructed vessel and around the outside of the vessel. At the end of 24 hours the cellular exudate became a mixture of polymorphonuclear neutrophiles, lymphocytes, and histiocytes. At this

3. Lillie, R. D.: Histopathologic Technic, Philadelphia, The Blakiston Company, 1948.

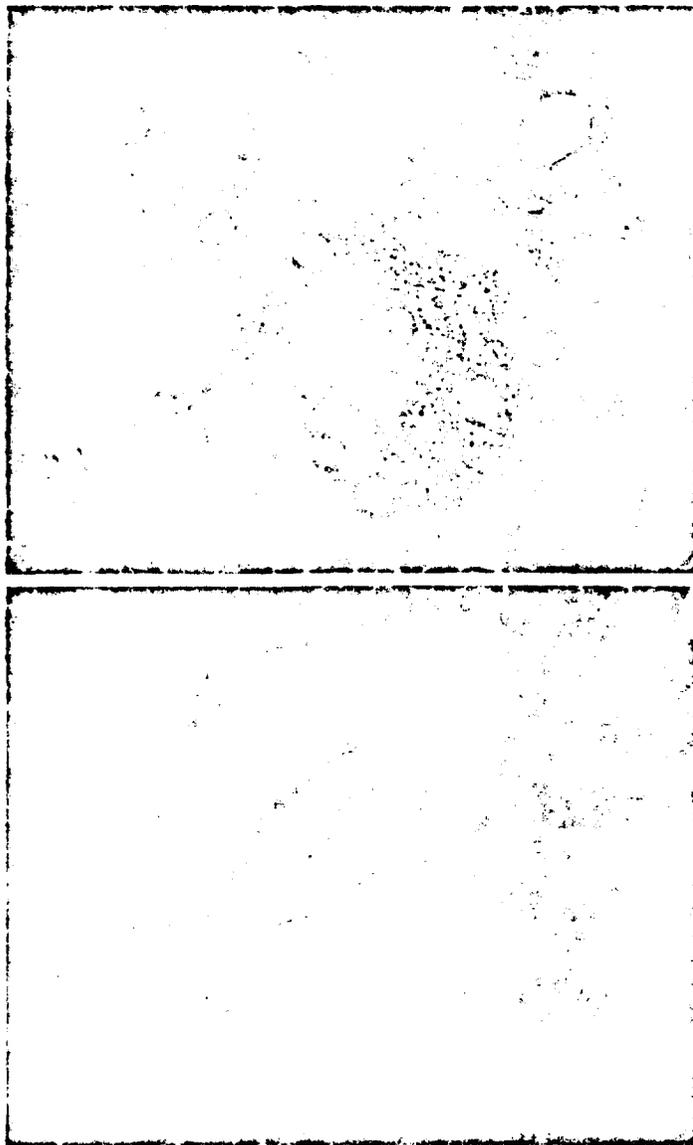


Fig. 2.—*A*, liver 24 hours after injection of cod liver oil. The lobules are delineated by numerous terminal branches of the portal vein, which are distended with oil. The vessels are surrounded by a cellular exudate. $\times 120$.

B, liver 24 hours after injection of cod liver oil. The cellular reaction around the vessels is composed of granulocytes in small number, lymphocytes, and histiocytes. The lumen is narrowed by proliferation of endothelial cells. $\times 420$.

time, too, at the site of the droplet the lumen of the vessel was narrowed by increased layers of flattened cells resembling endothelium (Fig. 2). Liver cords at the periphery of the lobule and in close relation to the occluded vessel showed evidence of coagulation necrosis involving a considerable portion of the lobule. At the borders of the necrotic areas the cytoplasm of the viable hepatic cells was markedly basophilic, and it was here that scattered small clusters of polymorphonuclear leucocytes were seen.

At the end of two days the intravascular space about the oil droplet was almost completely occluded by cells which appeared to be derived from endothelium. Some of these cells contained minute fat droplets in their cytoplasm. In the parenchyma the alterations were transient and moderate in extent. After the necrosis which was seen in the first 24 hours, the dead cells were no longer found at 48 hours, only the reticular structure remaining. Numerous mitoses of liver cells were visible at this time (Fig. 3*A*), and minute fat droplets were seen within the hepatic cells at the periphery of the lobules. Four days after oil injection the hepatic parenchyma was completely without alterations and remained so up to three months of observation.

The intravascular lesion, however, progressed until, one week after injection, numerous granulomas were present at the sites of oil-droplet obstruction (Fig. 3*B*). At this time the liver lobule was well delineated by interlobular inflammation and by interlobular vessels, which appeared increased in number as compared with the number found in control livers. Beneath the capsule the greatest number of granulomas was found, but here no definite pattern was discernible.

In the center of some of the granulomas there was a single large sudanophilic droplet, surrounded by macrophages and one or more foreign-body giant cells (Fig. 4*A*), which often contained engulfed sudanophilic material. The granuloma itself was fairly well demarcated, and at its periphery, outside the vessel and in its wall, were aggregates of lymphocytes, polymorphonuclear neutrophils, and histiocytic elements. At this time, too, reticulum and collagen preparations showed an obvious increase of fibers around the vessels, merging with the periphery of the granulomas and in some instances reaching to Glisson's spaces (Fig. 4*B*). In the subcapsular region these new strands of connective tissue occasionally surrounded small groups of hepatic cells to form pseudolobules. In areas other than the subcapsular region the fibrous tissue did not extend into the lobule. Rarely a focal granulomatous lesion was found on the capsular surface binding omental tissue to the liver. Elsewhere the capsule remained uninvolved.

At two weeks the granulomas appeared decreased in size and numbers. Lipid-filled macrophages were found at the site of granulomas in the newly formed fibrous tissue and also in Glisson's spaces. The size of the occluding droplet in the center of the granuloma was diminished and an increased number of macrophages with small sudanophilic droplets were visible.

Three weeks after injection few vascular lesions were present. In place of the granulomas one now found discrete scars (Fig. 5*A*) or accumulations of macrophages, many of which contained sudanophilic material in large vacuoles (Fig. 5*B*). The fibrous tissue and the cellular infiltrate about the interlobular vessels were diminished and the hepatic lobules were no longer clearly delineated. Here only sparse collections of lymphocytes remained. An increased number of lipid-filled macrophages were also present in Glisson's spaces.

At four weeks the liver was almost normal. Rarely a few strands of connective tissue, forming pseudolobules, might be found (Fig. 6*A*). Lipid-filled macrophages and lymphocytes were still numerous in Glisson's spaces. In some of the portal

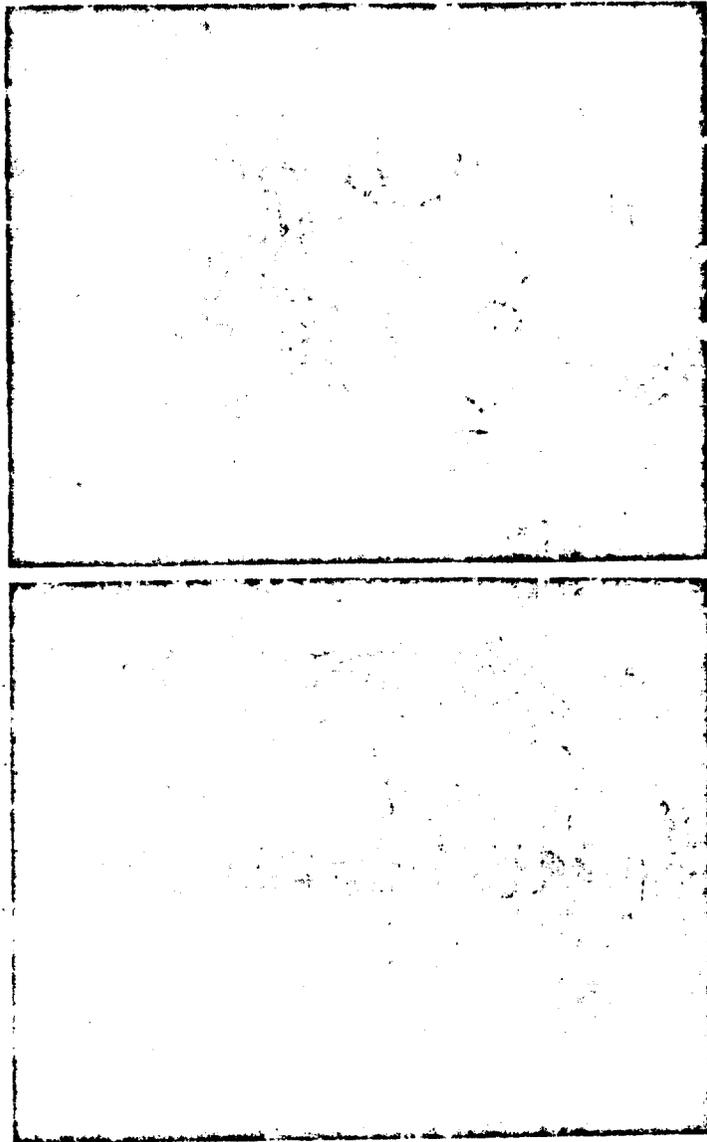


Fig. 3.—*A*, liver 48 hours after injection of cod liver oil, showing mitoses of liver cells. $\times 550$.

B, liver seven days after injection of cod liver oil. A granuloma with giant cells occludes a small branch of the portal vein. The stretched vessel wall can be distinctly recognized. $\times 500$.

vessels the lumens were narrowed by organized mural thrombi (Fig. 6*B*). A slight increase in the number of capillaries was discernible in Glisson's spaces.

PLATE II—LIVER CHANGES—INTRASPLENIC INJECTION OF FAT 63

Beyond four weeks the only evidence of the previous process was the presence of macrophages with large vacuoles in scattered areas corresponding to the sites of earlier granulomas and in Glisson's spaces.



Fig. 4.—*A*, liver seven days after injection of cod liver oil. The intravascular granuloma is composed of endothelioid foam cells and giant cells. The lumen of the vessel is filled by large oil droplets. $\times 500$.

B, liver 10 days after injection of cod liver oil. Fibrous strands are seen connecting the involved small vessels with the periportal connective tissue and in several places with sublobular veins. $\times 45$.

The lipid material in the phagocytes, which were first seen three weeks after intrasplenic injection of cod liver oil, was apparently not lost during the process

of embedding, and with the Ziehl-Neelsen technique it stained dark red. In hematoxylin-eosin-stained paraffin preparations many of the phagocytes contained fine reddish-brown granules. The Gömöri modification of the Prussian blue reaction revealed in many of the lipid-filled macrophages dark-blue irregular granules with

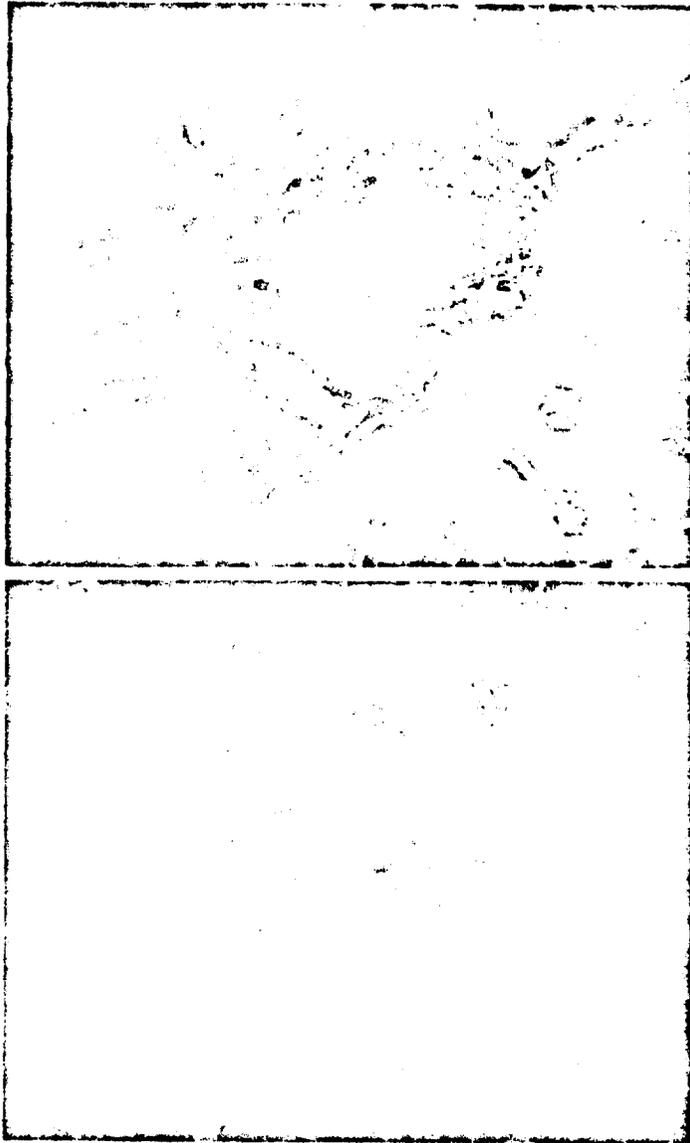


Fig. 5.—A, liver three weeks after injection of cod liver oil. A fibrous scar has replaced the granuloma, surrounded by young capillaries. $\times 430$.

B, liver three weeks after injection of cod liver oil. There are accumulations of phagocytes at the site of earlier granulomas. The cells contain sudanophilic and ceroid material. $\times 540$.

or without yellow-pigment granules. This was not a constant finding; all cells in the same liver with acid-fast lipid did not show a positive iron reaction, and in some

rats no iron was found despite the presence of numerous lipid-filled cells. Nor was there any correlation between the presence of iron and the age of the process.

In the liver of one animal three weeks after oil injection a single large oil droplet occluded the lumen of a vessel. This droplet was acid-fast with Ziehl Neelsen stain

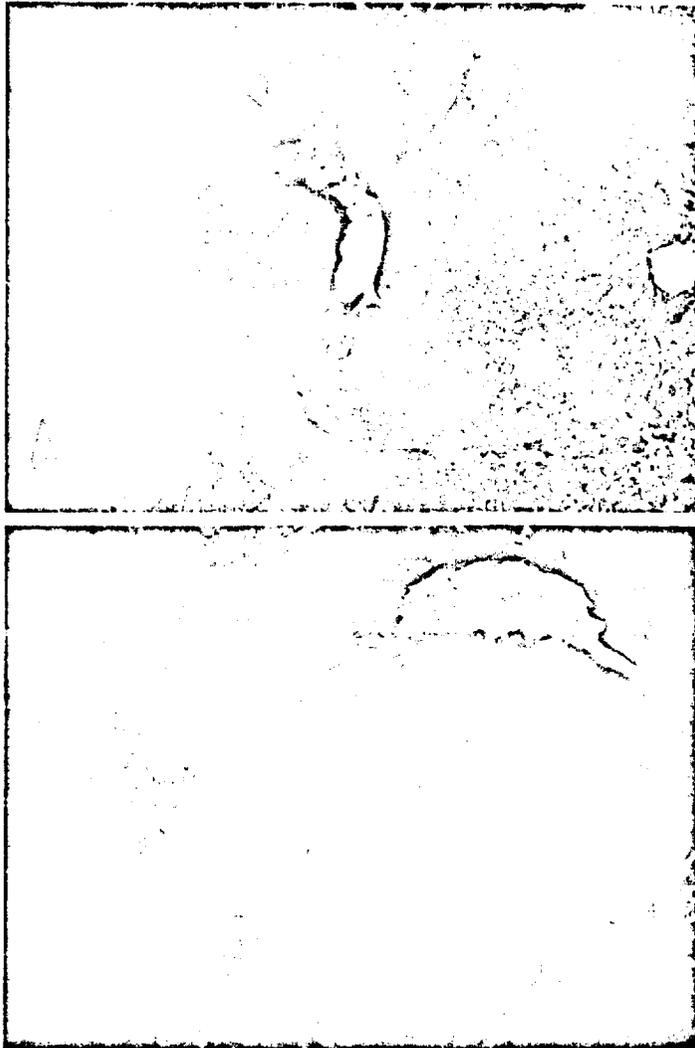


Fig. 6.—*A*, liver four weeks after injection of cod liver oil. Thin strands of connective tissue surround the lobules peripherally, extend to the spaces of Glisson's capsule and also to sublobular veins. Heidenhain's azocarmine modification of Mallory's connective tissue stain. $\times 100$.

B, liver four weeks after injection of cod liver oil, showing an organized mural thrombosis in a terminal portal ramification. Heidenhain's azocarmine Mallory stain. $\times 500$.

and was not dissolved during the process of embedding in paraffin. Phagocytic cells surrounding the globule contained minute acid-fast droplets.

The above series of events also occurred following the injection of 50% cod liver oil in acacia and following that of "neutralized" cod liver oil.

Five animals were splenectomized 24 hours after the intrasplenic injection of cod liver oil. The time sequence of lesions was visible in this group as described above for the non-splenectomized group.

Unsaturated Fatty Acids. The injection of 50% oleic acid or of 25 or 50% linoleic acid in acacia produced similar histological responses in the liver, varying only in a few minor details.

Three rats died within 24 hours after injection of 50% oleic acid, and four days after administration of 50% linoleic acid. The remaining animals were killed as indicated in the table.

The livers of animals which died within 24 hours after injection of an unsaturated fatty acid showed extensive necrosis. The necrotic areas were chiefly peripheral but occasionally extended toward the central vein. Sinusoidal dilatation and congestion were conspicuous, and hemorrhages were numerous, particularly into the

Interval Period and Number of Animals Killed After Intrasplenic Injection of Tested Material

	Days										Total Number of Animals
	0-15	1	2	4-5	7-10	14	21	28	60-120		
Cod liver oil.....	3	2	2	3	11	3	1	1	5		31
50% cod liver oil in acacia.....	1	2	2	2	2	2	1	1	0		13
"Neutralized" cod liver oil.....	0	4	3	2	3	2	1	0	0		15
Oleic acid 50%.....	4	3	3	2	4	1	1	0	0		18
Linoleic acid 50%.....	2	1	3	0	1	0	0	0	0		7
Linoleic acid 25%.....	0	3	0	1	3	1	1	1	0		10
Liquid petrolatum.....	0	2	2	1	1	1	0	1	0		8
Glycerol.....	0	2	3	2	2	1	0	0	0		10
Emulsion of rat body fat.....	1	4	2	2	2	0	0	0	0		11
Emulsion of fatty rat liver.....	0	2	2	2	2	1	1	0	0		10
Emulsion of normal rat liver.....	0	1	1	1	1	0	1	0	0		5
Cod liver oil; splenectomy 24 hr. later.	0	0	0	0	3	2	0	0	0		5
Total.....											143

necrotic regions. In the livers of the rats receiving linoleic acid there was a striking polymorphonuclear neutrophile infiltration in and around the necrotic foci as well as a scattering of these cells in the sinusoids. In the livers of the oleic acid series there was no polymorphonuclear neutrophile reaction.

At 18 hours the same extensive necrosis and focal hemorrhage were still visible. In addition, there were numerous mitoses throughout the remaining uninvolved parenchyma. Most of the viable hepatic cells contained small sudanophilic droplets, and in several areas larger fat droplets were lying free in sinusoids. In the oleic acid series the polymorphonuclear neutrophile response was sparse, while it was abundant in the linoleic acid group.

After 4 days the liver parenchyma appeared wholly intact, and the only vestige of injury was the presence of moderately increased numbers of lymphocytes in Glisson's spaces. Occasionally a variable number of lymphoid cells were seen within sinusoids without a particular pattern of distribution.

In all rats that survived a week or more, all the lobes of the liver were fused together, and the capsule was slightly opaque grossly but still glistening. Microscopically, the capsule was markedly thickened, at first by loose connective tissue and young fibroblasts, later by dense, less cellular fibrous tissue (Fig. 7*D*).

In none of the rats of this series was there any evidence of intra- or extra-vascular granulomas or of fibrous tissue increase in the parenchyma.

Glycerol and Liquid Petrolatum.—After a single intrasplenic injection of glycerol (0.3 cc.) there were at 24 hours small foci of hepatic cell necrosis near Glisson's



Fig. 7.—*A*, liver seven days after injection of linoleic acid. There is advanced thickening of the capsule by cellular connective tissue. $\times 450$.

B, liver seven days after injection of paraffin oil. Vessels are dilated by paraffin oil with no surrounding reaction. $\times 135$.

spaces and a moderate infiltration of polymorphonuclear neutrophiles was found in and around these foci. Twenty-four hours later the necrotic areas were decreased in number and size, the polymorphonuclear neutrophile infiltration was sparse, and

large macrophages appeared peripherally at the damaged sites. No fat droplets were visible either in vessels or in hepatic cells. Beyond two days the liver appeared almost normal, showing only an occasional Glisson's space containing small numbers of lymphocytes. In the two rats studied four days after injection of oil several areas beneath the capsule showed a few fine strands of connective tissue extending from the central vein through the lobule (Fig. 7B). Beyond this time there were no alterations.

Liquid petrolatum, in the first 24 hours, caused only dilatation of smaller branches of the portal tree beyond Glisson's sheath and necrosis of a few hepatic cells at the sites of oil obstruction. There were accumulations of lymphoid cells surrounding these dead cells but no reaction around the obstructed vessel. At four days and up to four weeks after administration, the liquid petrolatum droplets were seen in the same sites and still without surrounding reaction (Fig. 7B). No observations were made beyond this time.

Emulsions of Rat Tissues.—Rat body fat emulsified in acacia caused only minor histological changes. Within the first four days sudanophilic material was occasionally found in the lumina of large portal vessels lying in Glisson's spaces. Contiguous to these obstructed vessels a few hepatic cells were necrotic, and there was a slight infiltration of polymorphonuclear neutrophils around these cells. Beyond four days the liver appeared intact.

Injections of emulsions of normal rat liver resulted in occasional necrotic foci within the hepatic lobule which disappeared at 48 hours. In Glisson's spaces plasma and lymphoid cells accumulated in small numbers up to one week. Beyond this time the livers were normal.

Injections of emulsions of fatty rat liver were followed by no changes except a variable and slight increase of lymphoid cells in some of the portal spaces.

COMMENT

The present experiments were undertaken to analyze some of the factors likely to be effective in the production of fibrosis or cirrhosis in the liver. Fat substances were selected for injection because of their known irritating action on connective tissue and also because of their debated role in the pathogenesis of cirrhosis. Several authors have demonstrated a marked proliferation of collagenous tissue in the presence of unsaturated fatty acids and their soaps,⁴ and Tompkins⁵ observed the development of granulomas in subcutaneous tissues following local administration of phospholipids. Moreover, it was reported that, following hydrolysis of neutral fats in tissues in vivo, the inflammatory reaction was due to the presence of liberated fatty acids. The glycerol fraction was said to be without effect.^{4b} Glycerol and liquid petrolatum were used as control materials for the cod liver oil, fatty acids, and tissue emulsions. The purpose was to study the effects of vascular obstruction caused by inert oily substances.

4. (a) Hirsch, E. F.: Embolic Pulmonary Lesions Produced in Rabbits by Human Fat Containing Fatty Acids or Soaps, *Arch. Path.* **21**:765 (June) 1936; (b) Relation of the Chemical Composition of Lipids to Characteristic Tissue Lesions, *Arch. Path.* **31**:516 (April) 1941. (c) Hass, G. M.: Tissue Reactions to Natural Oils and Fractions Thereof, *Arch. Path.* **26**:596 (Nov.) 1938. (d) Hagerty.² (e) Lillie.³

5. Tompkins, E. H.: The Reaction of the Subcutaneous Tissues to the Acetone-Insoluble Lipoids from Beef Brains, *Bull. Johns Hopkins Hosp.* **79**:55, 1942.

This investigation revealed several significant facts. First, the different lipid materials were all effective in producing a greater or lesser degree of hepatic cell necrosis. But only cod liver oil elicited the granulomatous and fibrotic response. In explanation, this may possibly be ascribed to its chemical composition rather than to ischemia caused by oil-plugging of blood vessels. This conclusion seems plausible since no alterations were found in the livers of animals receiving injections of glycerol or liquid petrolatum, other than a transient and slight necrosis. According to Dr. F. A. Vandenheuvel,⁶ organic chemist, the Fisheries Research Board of Canada, about 98% of cod liver oil consists of a great variety of simple and mixed fatty acid glycerides, the rest being unsaponifiable matter. About 87% of the fatty acids are unsaturated, having from one to six double bonds. With such a mixture it is not possible to determine in the present experiments what fractions may be responsible for the observed changes.

Second, there was a temporal dissociation between the parenchymal necrosis and the development of vascular granulomas and perivascular fibrosis. The necrosis appeared in 24 hours and was completely absent four days after cod liver oil administration. The granulomas, however, were first seen between two and four days, progressed for a period of 10 more days, and then regressed. Accompanying their development and disappearance was a slight interlobular perivascular fibrosis which increased and then decreased with the granulomatous lesions.

The fibrotic response was apparently reversible. In the livers of all animals allowed to live from 10 to 14 days after administration of cod liver oil there was always an increase of the interlobular perivascular connective tissue. In the livers of all animals allowed to live up to four weeks or later, this was not seen. These observations corroborate the findings of others who have reported a regression in the fibrotic response after cessation of the employment of hepatic poisons.⁷

It has been shown in experimental protracted carbon tetrachloride poisoning of rats that beyond a certain period fibrosis was accompanied by transformation of the hepatic blood supply, and then the lesion became irreversible.⁸ In the present experiments the changes developed following a single dose of an irritant, and we cannot at present state whether repeated doses might have resulted in an irreversible type of fibrosis.

At the outset it was expected that a single intrasplenic injection would afford a continuous flow of fatty substances to the liver over a period of several weeks until the depot in the spleen was exhausted. We found, however, the same results in the liver when the spleen was removed 24 hours after intrasplenic injection of cod liver oil. Further, the same hepatic lesions developed in several rats, not reported here, which received repeated intrasplenic injections of the oil. These findings indicated

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that the hepatic damage occurred very soon after a single injection of oil and that little or no additional fatty substances were being supplied to the liver after the first one or two days.

Finally, only in the livers of rats given cod liver oil were phagocytes seen containing lipid material which was acid-fast, preserved during the process of embedding, and in some instances showing an iron-positive reaction. The intracellular material was most probably ceroid according to the definitions of Endicott and Lillie⁹ and Lee.¹⁰ It was likely derived from one or more of the components of cod liver oil other than oleic and linoleic acids, for injection of the latter unsaturated fatty acids did not result in the appearance of ceroid. Pinkerton¹¹ made similar observations in his study of cod liver oil pneumonia.

The present investigation supports to some extent the theory that hepatic cirrhosis is the result of selective irritation of the mesenchyma with or without simultaneous parenchymal injury. This idea was suggested years ago by Rössle¹² and more recently by Gillman and Chaikoff.¹³ There is, however, an obscure factor which seems to predispose to the development of intrahepatic fibrosis. Oleic and linoleic acids and liquid petrolatum cause a connective tissue increase elsewhere in the body. In our experiments oleic and linoleic acids produced a conspicuous capsular fibrosis of the liver. Yet, in the liver, despite the extensive parenchymal necrosis, there was no fibrous tissue response. Liquid petrolatum likewise produced no reaction within the liver. These observations suggest that the liver cell may be capable of inhibiting fibrous tissue formation, at least for some irritant substances.

SUMMARY

Cod liver oil injected into the spleen produced parenchymal injury in the liver within 24 hours. This injury disappeared in four days. Intravascular granulomas developed after two days, progressed in size and number up to two weeks, and finally disappeared after four weeks. Interlobular perivascular fibrosis developed within 10 days and regressed after two weeks. Ceroid in macrophages was first seen three weeks after administration of cod liver oil and was present up to three months, the period of observation.

Unsaturated fatty acids (linoleic and oleic), oily substances (glycerol and liquid petrolatum), and emulsions of rat body and liver fat were also injected into the spleen. These agents caused a variable and transient parenchymal injury without mesenchymal response. Ceroid was not found in the liver after the administration of these substances.

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**MITOCHONDRIAL SWELLING AND UNCOUPLING
ACTIVITY OF LONG-CHAIN FATTY ACIDS.**

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In 1960 SCHOLEFIELD¹ reported the inhibition of phosphorylation in the cancer cells by fatty acids and in 1956 LEHNINGER² found uncoupling oxidative phosphorylation and swelling action³ of fatty acids and of endogenous uncoupling factor such as unsaturated long-chain fatty acids, on mitochondria. In 1956 PRESSMAN and LARDY⁴ 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 HOGEBOM and SCHNEIDER's method⁵. 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 LEHNINGER⁴. 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 contraction of the swollen mitochondria induced by fatty acid⁶ 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 method⁷ 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), 0.30 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 ³²P 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 μ c. 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 \times g, washed 3 times with 0.25 *M* sucrose solution to eliminate the contamination of absorbed ³²P. The acid soluble phosphate compounds in mitochondria were extracted with 3 ml of 5 per cent trichloro-acetic acid for 30 minutes at 0°C and centrifuged for 10 minutes at 1700 \times 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* H₂SO₄ for 10 minutes at 100°C and the quantity and radioactivity of ³²P were estimated by the TAKAHASHI's method⁷.

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's⁸. 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 respiration and oxidative phosphorylation in the presence of succinate.

The incorporation of ³²P 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 μ c of ³²P, 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 (\times 4, 200–400 meshes) of formate type and eluated with formic acid and ammonium formate according to the method of TERADA⁹.

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 carbon₁₄ myristate at the concentration of 0.1 *mM*. The swelling activity of C₁₂ laurate and C₁₆ palmitate are lower than that of myristate and the lowest are of C₁₈ stearate and C₂₂ 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 C₁₈ fatty acids (sodium salts) on the mitochondrial swelling. The test system is as in Fig. 1 and expressed 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	9.8	8.0
Oleic	9, cis	50.2	41.2	83.8	69.8
Elaidic	9, trans	10.3	8.5	60.0	49.2
Richinoleic	9,	56.1	40.6	76.7	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

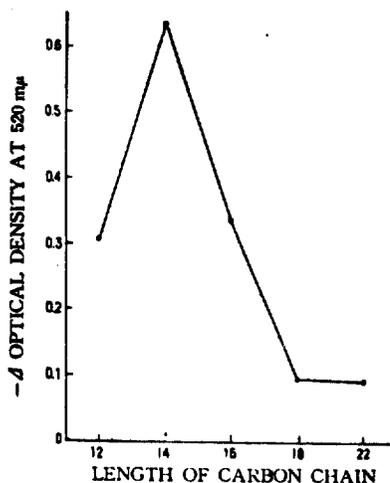


Fig. 1. The mitochondrial swelling action of saturated fatty acids (sodium salts) as function of carbon chain length. A medium of 5.0 ml of 0.15 M KCl-0.02 M Tris pH 7.4 was added to each tube, containing 0.1 mM of fatty acid. The changes of optical density were measured at 520 m μ at 37°C 20 minutes after the addition of washed rat liver mitochondria derived from 50 mg whole liver. The data are estimated with $-\Delta$ optical density at 520 m μ .

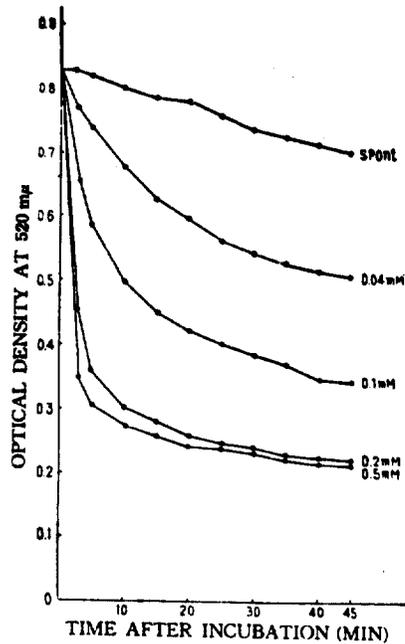


Fig. 2. Effect of various concentrations of sodium oleate on the swelling of mitochondria. The medium consisted of 5.0 ml of 0.15 *M* KCl-0.02 *M* Tris, pH 7.4. The amount of oleate shown was added, and optical absorbancy changes were measured at 520 μ at 20°C after the addition of washed rat liver mitochondria derived from 100 mg whole liver.

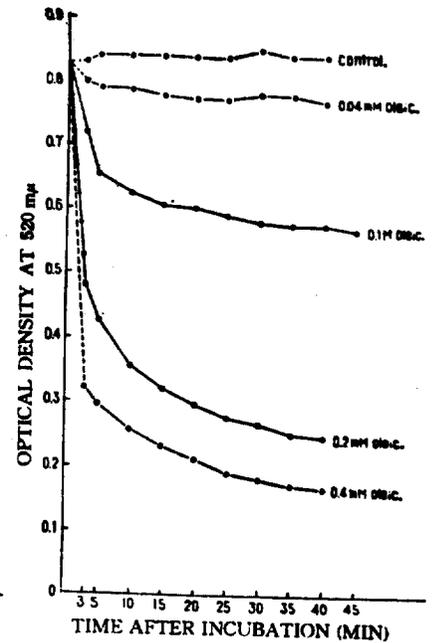


Fig. 3. Effect of various concentrations of sodium oleate on the swelling of mitochondria. The medium consisted of 5.0 ml of 0.25 *M* sucrose-0.02 *M* Tris, pH 7.4. The amount of oleate shown was added, and the reaction followed at 20°C as shown in Fig. 2

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 LEININGER⁸. He⁸ 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 factor¹⁰ 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 COOPER¹¹ reported the rate of swelling varies due to tissues which mitochondria are prepared from, and ARCO^{12,13} in 1960 stated that rat liver mitochondrial swelling induced by various agents was reduced in the process of

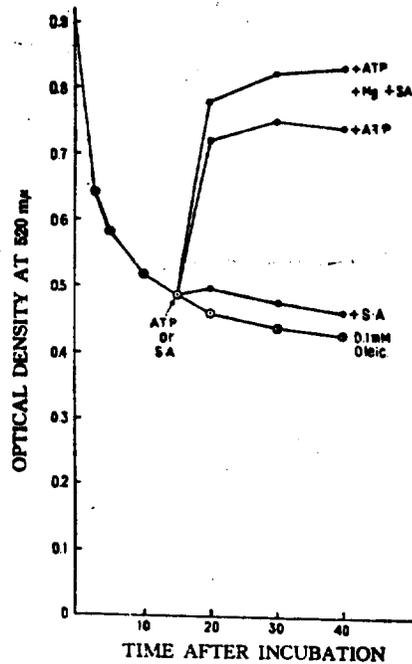


Fig. 4. Requirements for reversal of swelling induced by sodium oleate. The incubation mixture contained of 5.0 ml of 0.15 *M* KCl-0.02 *M* Tris (pH 7.4), 0.1 *mM* sodium oleate and 100 mg tissue equivalent mitochondria. Additions were 5 *mM* ATP, 5 *mM* MgCl₂ and 3 mg per ml of bovine serum albumin. 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 their 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.

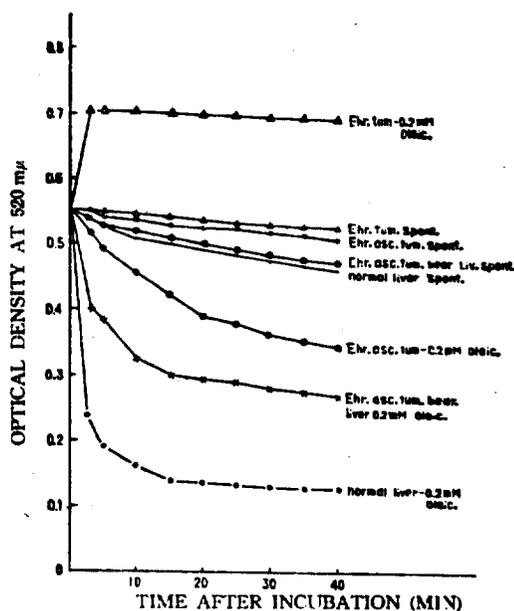


Fig. 5. Effect of sodium oleate (0.2 mM) on the mitochondrial swelling of mouse liver (50 mg), Ehrlich tumor bearing mouse liver (50 mg) and Ehrlich ascites tumor cells (250 mg) (solid and ascites). The details as in Fig. 2.

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 m μ .

Substrate	Non	Amytal ($1 \times 10^{-3}M$)	Ant. A (10 γ , 5ml)	Azide ($5 \times 10^{-3}M$)	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)	86.2 (I)	
Succinate (")	85.2 (A)	86.7 (I)	98.0 (I)	96.2 (I)	85.0	
Glutam. (")	70.0 (A)	74.2 (I)	80.8 (I)	83.6 (I)	77.0 (I)	
α -KG* (")	64.9 (A)		81.3 (I)			
Oleate($8 \times 10^{-5}M$)	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 previously¹⁹, 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 $\Delta 10\text{P}$ 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 experiment¹⁹. This phenomenon is observable only in the intact mitochondria having the respiratory control²⁰.

By oxygraphic measurement of respiration and oxidative phosphorylation,

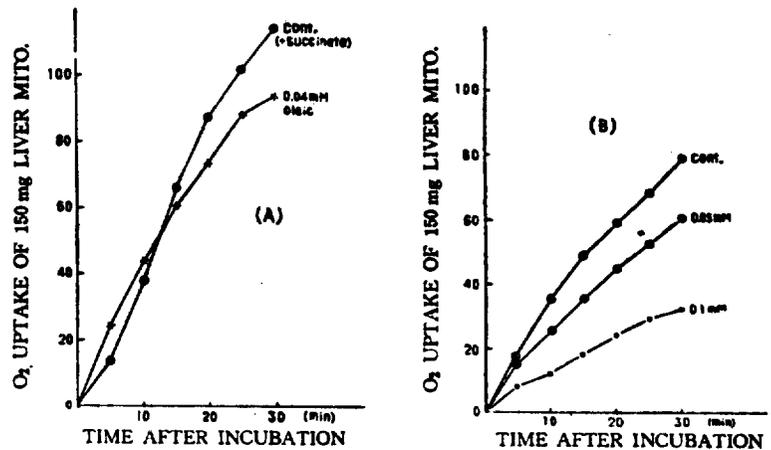


Fig. 6. Effect of sodium oleate on the respiration of rat liver mitochondria. Detail of incubation system as in the text.

(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 $\Delta 10\text{P}$ fraction of rat liver mitochondria. Detail of incubation mixture as in the text.

	Ratio of RA of Pi	Ratio of RA of $\Delta 10\text{P}$	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	83.4

RA : relative activity

SA : specific activity

the stimulated respiration under the existence of succinate and uncoupling 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 $m\mu$ 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 ($m\mu$ atom)	P/O	O ₂ consumption ($m\mu$ atom)	P/O
Lauric acid	30.0	1.54	45.0	1.05
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	1.82	17.5	1.82
Oleic acid	30.0	1.67	50.0	0.01
Eleidic acid	30.0	1.82	32.5	1.54

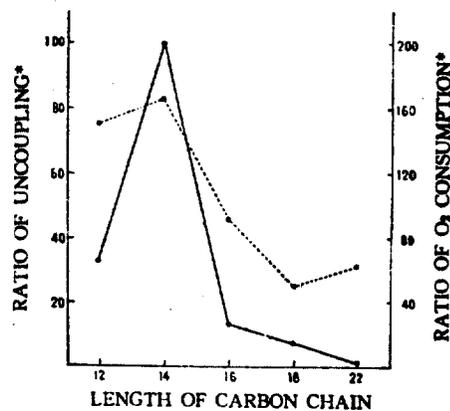


Fig. 7. Effect of various saturated fatty acids (sodium salts) on the respiration and phosphorylation of rat liver mitochondria. The medium contained as described in the text. (0.08 mM of sodium oleate and 100 mg tissue equivalent of mitochondria).

* Ratio of uncoupling $\left(1 - \frac{p/o \text{ of after treatment}}{p/o \text{ of before treatment}}\right) \times 100$ -----

* Ratio of O₂ consumption $\frac{O_2 \text{ up take of after treatment}}{O_2 \text{ up take of before treatment}} \times 100$

The inhibition of ³²P incorporation into Δ10P fraction of mitochondria by sodium oleate: From the data of uncoupling oxidative phosphorylation by fatty acid, the effect of oleic acid on the ³²P incorporation into acid soluble organic phosphate compounds is examined to reveal which fraction

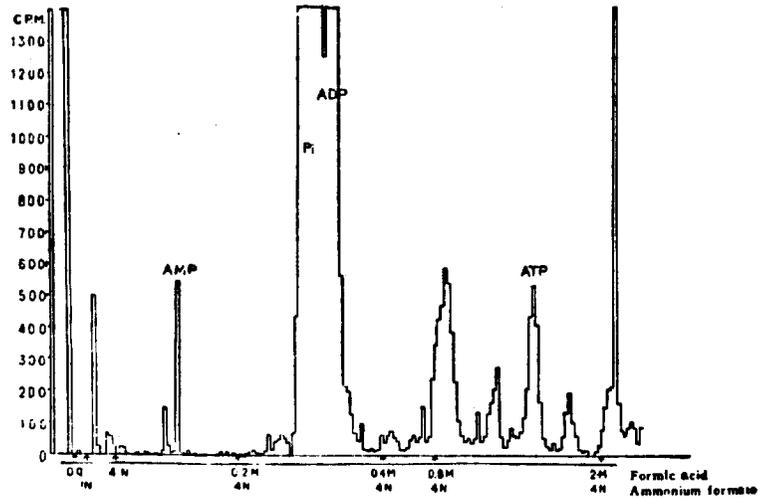


Fig. 8. Incorporation of ^{32}P into acid soluble phosphorous compounds of rat liver mitochondria (500 mg) after incubation 30 minutes at 25°C . The incubation mixture consisted with 3 ml of mitochondrial suspension, 1 ml of KRP solution (containing $100\ \mu\text{C}$ of ^{32}P), 1.0 ml of 0.2M sodium succinate and 5.0 ml of 0.15 M KCl-0.02M Tris buffer solution (pH 7.4).

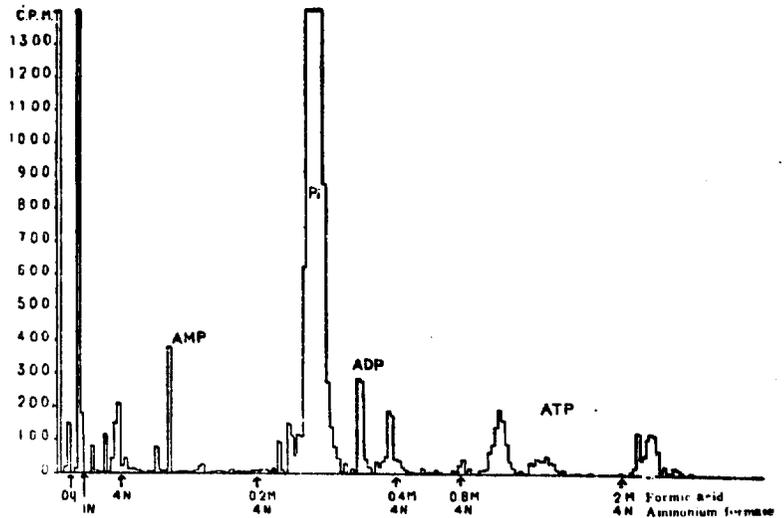


Fig. 9. The Effect of sodium oleate on the incorporation of ^{32}P into acid soluble phosphorous compounds of rat liver mitochondria. Incubation mixture containing 0.5 mM sodium oleate.

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^{2,5,6,14-23} concerning the relation among the respiration, oxidative phosphorylation and swelling of mitochondria, especially noteworthy one is the recent report of PACKER⁴ 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 stimulator of latent ATPase activity. In this experiment it has been clarified that the extent of uncoupling action is parallel to that of mitochondrial swelling action of fatty acid as a function of the carbon chain length. This finding is similar to the stimulation of latent ATPase activity by various fatty acids as reported by PRESSMAN and LARDY⁴ 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 LEININGER²⁵ 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 indistinguishable. The experiments are being conducted to test whether 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 OHNISHI²⁶ 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 RACKER²⁷ also reported the coupling factor containing the latent ATPase activity. The mitochondrial membrane consists of structural protein, lipids and elementary particles⁸. 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.

CORWIN¹⁴ 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^{29,30}. 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^{20,18}, HULSMANN¹⁸ and PRESSMAN⁴: 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 metabolism in natural system. Namely, the usual endogenous uncoupling factor or endogenous respiratory inhibitor contains some isooctane soluble fatty acids perhaps 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^{2,3}.

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, palmitate, stearate and behenate in saturated fatty acid and linoleate, linolenate, ricinoleate 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, Mg²⁺ 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 ³²P into ATP, ADP, GTP and UDPG in mitochondria.

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REDUCTION OF CARCINOGEN-INDUCED BREAST CANCER IN RATS

BY AN ANTI-FERTILITY DRUG*

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ENDOCRINE status greatly affects mammary cancer induction by chemical carcinogens. For example, Huggins and associates¹ have demonstrated that although female Sprague-Dawley rats, age 55 days, developed mammary cancer readily after single doses of 7,12-dimethylbenz[a]anthracene (DMBA), the incidence of the tumors produced and the latent period were highly susceptible to endocrine control²⁻⁸.

In view of the present interest in the possible effects of oral contraceptives on endocrine-related cancers, the DMBA-induced mammary cancer system seemed an ideal model to study the influence of these drugs. Thus, rats were placed on two levels of a contraceptive (Enovid) for a moderately long period of time. A single dose of the carcinogen was superimposed on this treatment. We found a reduction in the total incidence as well as in the tumor multiplicity in animals given the anti-fertility drug, as compared to vehicle-treated controls.

Experimental Procedures

Groups of 20 female Sprague-Dawley rats, age 40 to 45 days, weighing approximately 150 g, were given by gastric tube daily for 45 days 3.0 or 0.3 mg of crystalline Enovid (composed of 1.5% mestranol and 98.5%

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norethynodrel, kindly supplied by G. D. Searle and Co., Chicago, Illinois) suspended in 1 ml of sesame oil per dose⁹. Ten days after the initiation of the contraceptive treatment the rats received a single oral dose of 15 mg of DMBA in 1 ml of sesame oil. The controls consisted of a group of 20 rats given a single dose of 15 mg of DMBA, of 2 groups of 10 rats administered daily doses of 3.0 and 0.3 mg, respectively, of Enovid for 45 days, and of 1 group of 20 rats on sesame oil.

In addition to these 6 groups, three additional sets of 5 rats each were fed 3.0 or 0.3 mg Enovid per rat, or 0.3 mg Enovid plus the subsequent single dose of 15 mg DMBA. The functional response of the endocrine system to the treatment was determined by examination of vaginal smears, stained with Wright's stain, performed daily for a 5-day period every other week.

The animals were weighed weekly and thoroughly examined for the appearance of mammary tumors or other grossly apparent lesions. Animals which appeared ill for any reason were observed twice daily and killed when death seemed imminent. After 9 months, all animals were carefully necropsied, gross lesions were noted, and select tissues were examined microscopically after fixation in 10% formalin and conventional histologic processing.

Results

The dosage of Enovid was selected on the basis of preliminary experiments. With doses of 0.005 mg per 150 g rat the estrus cycle was usually but not always arrested 5 days after the treatment began. At doses of 0.05 and 0.5 mg, respectively, the cycle was interrupted 3 to 5 days, and 1 day later. In the final studies with doses of 0.3 and 3.0 mg, the microscopic examination of the smears indicated that the estrus cycle was effectively halted in all rats throughout the Enovid, or Enovid plus carcinogen feeding period, but returned to normal shortly after the cessation of Enovid administration. DMBA failed to restore the estrus cycle suggesting that Enovid exerts a

pronounced effect on the endocrine system. Stern *et al.*¹⁰ reported that vaginal cycling was resumed after DMBA administration to androgen-sterile rats.

A level of 0.5 mg per day per rat of Enovid fed to adult female Wistar strain rats for 4 months appeared to increase liver weights as compared to controls¹¹. In our series Enovid was given for only 45 days and autopsies were performed 7.5 months later. No significant differences in the liver weights in any of the groups were observed.

A single dose of 15 mg DMBA yielded an increasing incidence of mammary tumors which reached 100% after about 6 months (Table 1). The multiplicity also rose and reached an average of 5.8 masses per rat at the end of the planned experimental period of 9 months. Histopathologic examination of the tumors indicated that there were several types, and even the same tumor nodule exhibited areas of varying morphologic aspect (*see also* 12-14). With DMBA most of the lesions were lobular carcinomas, grades 0 to 2, but giant fibroadenomas were also frequent. Additionally, there were numerous fibroadenomatous hyperplasias, some of which were the lactating type.

Administration of the lower level of Enovid tended to inhibit slightly the incidence of mammary cancer, measured by the percentage of rats affected and by the multiplicity of the grossly detectable active centers. As compared to rats fed DMBA alone, a larger percentage of the rats survived for the entire 9 months, as a result of a lower fatality rate presumably associated with the presence and growth of mammary tumors. The higher level of Enovid had a more pronounced effect. Thus, the tumor multiplicity was reduced to 3.3 with the 3 mg level of Enovid, from 5.8 in the DMBA controls. Also, only 56% of the animals had histologically verified mammary carcinoma versus 86% in the DMBA controls (Table 2). The anti-fertility drug also delayed tumor appearance which was first noted at the 120-day point, as compared to 60 days in the rats without drug.

TABLE 1
Per Cent Survivors, Per Cent Survivors with Masses, and Mean Number of Subcutaneous Masses
by Palpation Following Administration of DMBA and Enovid^{a/}

Days after First Exposure	DMBA			DMBA + 0.3 mg Enovid			DMBA + 3 mg Enovid		
	% Survivors	% Survivors with Masses	Mean No. of Masses	% Survivors	% Survivors with Masses	Mean No. of Masses	% Survivors	% Survivors with Masses	Mean No. of Masses
0	100	0	0	100	0	0	100	0	0
30	100	0	0	100	0	0	100	0	0
60	100	25	0.3	100	15	0.2	100	0	0
90	100	45	1.0	100	35	0.4	100	0	0
120	100	70	1.8	95	63	0.4	100	30	0.3
150	90	83	2.7	95	68	1.0	95	47	0.6
180	70	100	3.1	85	82	1.6	95	68	1.3
210	60	100	4.4	80	88	2.4	90	61	1.7
240	50	100	4.5	80	94	3.0	85	65	2.0
270	40	100	5.8	80	100	4.6	75	80	3.3

^{a/} No masses were found in the group of 10 rats given 0.3 mg doses of Enovid alone and 1 was observed 270 days after first exposure in the group of rats fed 3 mg.

TABLE 2
Histopathological Diagnosis of Mammary Tumors in DMBA- and Enovid-Treated Rats

	DMBA 15 mg	DMBA 15 mg + 0.3 mg Enovid	DMBA 15 mg + 3.0 mg Enovid	Enovid 0.3 mg	Enovid 3.0 mg	Sesame Oil Controls	
No. of rats examined	14	18	18	9	10	19	132
No. with tumor	14	18	14	1	3	1	5
No. of carcinomas	26 (12) ^{a/}	19 (12)	17 (10)	0	3 (1)	0	3 (1)
fibroadenomas	7 (6)	8 (7)	6 (5)	0	0	1 (1)	1 (1)
hyperplasia	19 (7)	43 (14)	24 (9)	1 (1)	2 (2)	0	5 (4)
xanthoma	1 (1)						
Total no. of masses	53	70 ^{b/}	47 ^{c/}	1	5 ^{d/}	1	9
Percent of rats with carcinoma	86	67	56	0	10	0	0.8

^{a/} The number in parentheses represents the number of rats in which the lesions occurred.

^{b/} One lung adenocarcinoma and 6 cases of cystic adrenals were also seen in this group.

^{c/} Two cystic adrenals and 1 case of squamous metaplasia of the fallopian tube were found.

^{d/} One kidney carcinoma, two cases of cystic ovaries and 1 case of cystic changes in the adrenals also occurred. The kidney tumor was found in the rat which had the 3 mammary carcinomas.

Microscopic fibroadenomatous hyperplasia occurred in one rat on 0.3 mg of Enovid, killed after 299 days, and in two rats on the 3.0 mg level. One rat on the 3.0 mg dose had 3 lobular breast carcinomas and a kidney carcinoma. Cystic changes in the ovary or adrenals also were seen in this group.

Of 20 sesame oil control animals, only one, killed after 237 days, had a large fibroadenoma. In a separate group of 132 controls which were part of a simultaneous series of related experiments, there were 4 rats with fibroadenomas or fibroadenomatous hyperplasia, while 1 rat had multiple tumors, namely 3 mammary carcinomas and 1 fibroadenomatous hyperplasia (Table 2). In an earlier study⁹, 84 controls fed sesame oil showed 1 case each of breast hyperplasia, carcinoma, pulmonary carcinoma, and basal hyperactivity of the bronchus.

Discussion

The present experiments demonstrate that treatment with an oral contraceptive drug at 2 dose levels, superimposed on a single carcinogenic challenge, resulted in a moderation in the carcinogenic effect to the mammary gland of a sensitive strain of rat. The higher dosage was considerably more effective than the lower one, even though both affected the endocrine system, as revealed by an absence of estrus cycles. The inhibition of tumor development in the mammary gland may rest on a purely hormonal mechanism, but it is also possible that Enovid altered the effective level of carcinogen in the breast (see 3, 15) by biochemical means, for example by an increased titer of detoxifying enzymes in the liver or even in the mammary gland.

A more dramatic reduction in carcinogenicity was found by Huggins and Yang¹⁶ who administered 20 μ g of estradiol plus 4 mg progesterone daily for 30 days 50 days after a single dose of carcinogen. With this regimen even existing lesions regressed. In our case a more prolonged period of Enovid administration might have possibly led to a more pronounced effect. Gruenstein *et al.*⁷, however, found that continuing Enovid administration at 3 mg per day

in carcinogen-pretreated rats and removing the early mammary tumors surgically, did not prevent the development of additional new tumors or tumor recurrences. Furthermore, the same investigators¹⁷ saw no effect when Enovid was administered together with or subsequent to carcinogen. However, the carcinogen treatment was quite extensive and may have overwhelmed the weaker inhibiting effect of the anti-fertility agent.

McCarthy¹⁸ also observed no effect on mammary tumor formation by a single dose of carcinogen when the contraceptive drug was given for 10 days prior to carcinogen, although with small dosages of drug, an apparent enhancement of the carcinogenic process was observed. In our tests hyperplastic mammary glands were more numerous in rats given the lower dosage of Enovid and DMBA. Preliminary data by Fletcher *et al.*¹⁹ also suggest that Enovid may have accelerated the development and growth of mammary tumors but they state that "after initial, very rapid growth rate there was some diminution in the number of tumors in the treated group." They indicated the need for studies with a longer period of drug administration. The results we have obtained demonstrate that with the protocol used here, at a high dose level compared to that used in clinical practice²⁰⁻²², the oral contraceptive treatment does not lead to an enhancement but rather to a reduction in the carcinogenicity of DMBA.

Summary

Beginning with female Sprague-Dawley rats 40 days old, 0.3 and 3.0 mg of Enovid in sesame oil were given daily for 45 days. A single dose of 15 mg of 7,12-dimethylbenz(a)anthracene in oil was fed by gastric tube to the rats at the age of 55 days. There were controls on carcinogen alone, oil, and Enovid. Rats treated with carcinogen and the higher level of Enovid showed a reduced incidence of mammary tumors and a lower multiplicity of the tumors, as compared to controls on carcinogen alone, in a period of observation of 9 months.

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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND
INHIBITION OF ATP-P_i EXCHANGE BY
A SUBSTANCE FROM INSECT MITOCHONDRIA

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SUMMARY

Human and bovine serum or plasma albumins, and to a smaller degree β -lactoglobulin, stimulate oxidative phosphorylation and the ATP-P_i exchange reaction in mitochondria from larvae of the wax-moth, *Galleria mellonella* L. (Lepidoptera). This effect has been shown to be due to removal by these proteins of an uncoupling agent that is present in isolated insect mitochondria. It has been demonstrated that this uncoupling agent contains fatty acids, of which palmitic, stearic, oleic, linoleic and linolenic acids have been identified by paper chromatography.

INTRODUCTION

It is known that oxidative phosphorylation in isolated insect mitochondria is usually less efficient than in mitochondria from mammalian liver or heart. The yield of phosphorylation in insect tissue particles can be increased by the addition to the incubation medium of serum albumin^{1,2} or of some other proteins³. This effect is best seen with sarcosomes from thoracic muscles of the housefly in which no phosphorylation is observed until bovine serum albumin is added¹. An explanation has been proposed¹ according to which this stimulatory effect may be due to preservation of mitochondrial integrity by colloidal substances, as suggested by WATANABE AND WILLIAMS⁴. This explanation has recently appeared to be unsatisfactory since it has been shown⁵ that, among a variety of proteins tested, only a limited number has the stimulatory effect on oxidative phosphorylation in housefly sarcosomes.

In a preliminary communication⁶ we described a stimulatory effect of serum albumin on oxidative phosphorylation and the ATP-P_i exchange reaction in mitochondria from wax-moth larvae and were able to show that this effect is due to the binding of albumin with an uncoupling agent. In this paper more details of these experiments are reported and evidence is presented that the uncoupling substance contains a mixture of fatty acids.

Abbreviations: P_i, orthophosphate; AMP, adenosine 5'-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; pCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose as described by MYERS AND SLATER⁷. Wax-moth mitochondria were obtained from whole fully grown larvae of the wax-moth, *Galleria mellonella* L. by the same procedure: mitochondria used in experiments on ATP-P₁ exchange were isolated and suspended in 0.25 M sucrose; mitochondria used for measurements of phosphorylation were isolated in 0.25 M sucrose containing 5 mM ATP and were finally suspended in 0.25 M sucrose containing 1 mM ATP, as recommended by HOLTON *et al.*⁸ for heart sarcosomes.

Oxidative phosphorylation was investigated in a medium containing: KCl, 20 μ moles; MgCl₂, 6 μ moles; EDTA, 2 μ moles; phosphate buffer, pH 7.2, 10 μ moles; Tris-HCl buffer, pH 7.2, 18 μ moles; AMP, 0.5 μ mole; ATP, 0.2 μ mole; sodium succinate or glutamate, 8 μ moles; glucose, 10 μ moles; sucrose, 50 μ moles; hexokinase, 200 Kunitz-McDonald⁹ units; mitochondria containing 2-4 mg protein. Final volume was 1.0 ml; temperature, 25°; incubation time, 20-40 min. Oxygen uptake was measured in micro-flasks (about 5 ml capacity) of a conventional Warburg apparatus; phosphorylation was determined by measuring the amount of either hexose monophosphate formed¹⁰ or orthophosphate disappeared¹¹ (both values appeared to be consistent within the limits of experimental error).

ATPase activity was determined as described by MYERS AND SLATER⁷ in a medium containing: KCl, 112.5 μ moles; MgCl₂, 4.5 μ moles; EDTA, 1.5 μ moles; ATP, 3 μ moles; sucrose, 162.5 μ moles; mitochondria containing about 1 mg protein. Total volume was 1.5 ml; pH 7.4; incubation time, 15 min; temperature, 20°.

ATP-P₁ exchange was investigated using a slightly modified incubation medium of HÜLSMANN *et al.*¹² which contained in a total volume of 1.0 ml: KCl, 100 μ moles; MgCl₂, 4 μ moles; EDTA, 1.5 μ moles; Tris-HCl buffer, pH 7.5, 34 μ moles; phosphate buffer, pH 7.5, containing about 10⁶ counts/min ³²P, 3 μ moles; ATP, 5 μ moles; sucrose, 12.5 μ moles; mitochondria containing about 1 mg protein. Incubation time was 15 min at about 20°. [³²P]ATP formed was determined as described by NIELSEN AND LEHNINGER¹³. The values expressed as μ atoms P exchanged/mg mitochondrial protein/h are corrected for ATPase activity, assuming that the rates of exchange reaction and of hydrolysis of ATP are linear within the time of experiment.

Preliminary experiments on ATP-ADP exchange were carried out with the same incubation medium as for ATP-P₁ exchange except that ³²P₁ was omitted and 3 μ moles ADP containing about 150,000 counts/min [³²P]ADP were added. The medium contained 0.01 M NaF. Incubation time was 5 min at 20°. The nucleotides were separated on a Dowex-2 column and the radioactivity was measured with a thin-window Geiger-Müller counter.

Protein content in mitochondrial suspensions was determined by the biuret method according to the procedure of CLELAND AND SLATER¹⁴.

Chromatography of fatty acids was carried out on Whatman No. 3 filter paper impregnated with liquid paraffin¹⁵. Spots of fatty acids were made visible by the copper acetate-potassium ferricyanide procedure and those of unsaturated fatty acids by alkaline permanganate treatment as described by KAUFMANN¹⁵.

AMP and ADP were products of Light & Co. Ltd.; ATP was obtained from Fluka AG or was prepared from rabbit muscles¹⁶. Protein preparations were obtained as follows: bovine plasma albumin fraction V and protamine sulfate, from Light & Co.

Ltd.; crystalline bovine serum albumin, bovine β -lactoglobulin and cytochrome *c*, from Nutritional Biochemicals Corporation; human serum albumin fraction V, human γ -globulin and human haemoglobin, from the Institute of Haematology in Warsaw. Anserine and carnosine were kindly offered by Prof. S. E. SEVERIN, Moscow. Hexokinase (crude, type II) was obtained from Sigma Co.

[32 P]ADP was prepared as described by COLOWICK AND KAPLAN¹⁷.

RESULTS

Effect of proteins on oxidative phosphorylation and on the ATP-P_i exchange reaction in wax-moth mitochondria

Values of the P:O ratio as low as 0.2 were obtained with succinate if no protein was added to the incubation medium. With serum or plasma albumin, however, the efficiency of phosphorylation was increased considerably and P:O ratios as high as 1.4 were obtained. Among a number of proteins tested (Table I) only β -lactoglobulin was found to have a similar although smaller effect.

Addition of human or bovine serum albumin not only strongly stimulated the esterification of inorganic phosphate, but also increased O₂ uptake by the respiring mitochondria; mean values from 10 experiments were: 2.80 μ atoms O₂/mg mitochondrial protein/h if no albumin was added and 3.78 μ atoms O₂/mg protein/h in the presence of 9 mg albumin.

Further experiments showed that serum and plasma albumins and β -lactoglobulin but none of the other proteins tested appreciably increased the ATP-P_i exchange catalysed by wax-moth mitochondria (Table I).

Fig. 1 illustrates the effect of various amounts of serum albumin on the P:O ratio and ATP-P_i exchange. It can be seen that as little as 1 mg albumin/ml incubation medium produces half maximum stimulation of the exchange reaction, whereas higher concentrations of albumin are necessary to increase the net phosphorylation.

TABLE I
EFFECT OF PROTEINS ON PHOSPHORYLATION COUPLED TO OXIDATION OF SUCCINATE AND ON ATP-P_i EXCHANGE IN MITOCHONDRIA FROM WAX-MOTH LARVAE

Protein added*	Oxidative phosphorylation (P:O)	Exchange reaction (μ atoms P/mg mitochondrial protein/h)
None	0.2 (10)	0.1 (10)
Crystalline bovine serum albumin	1.1 (5)	1.1
Bovine plasma albumin, fraction V	1.3 (5)	2.2 (8)
Human serum albumin, fraction V	1.4	1.9 (3)
Egg albumin	0.1	0.1
Human serum γ -globulin	0.3	0.2
β -Lactoglobulin (bovine)	0.4 (2)	2.1 (2)
Haemoglobin (human)	0.1	0.1 (2)
Cytochrome <i>c</i>		0.1
Casein	0.0	0.0
Insulin	0.1	0.2
Protamine sulfate		0.1

Number of experiments, if more than one, is indicated in parentheses.

* 9.0 mg were added in phosphorylation tests and 1.5 mg in ATP-P_i exchange experiments.

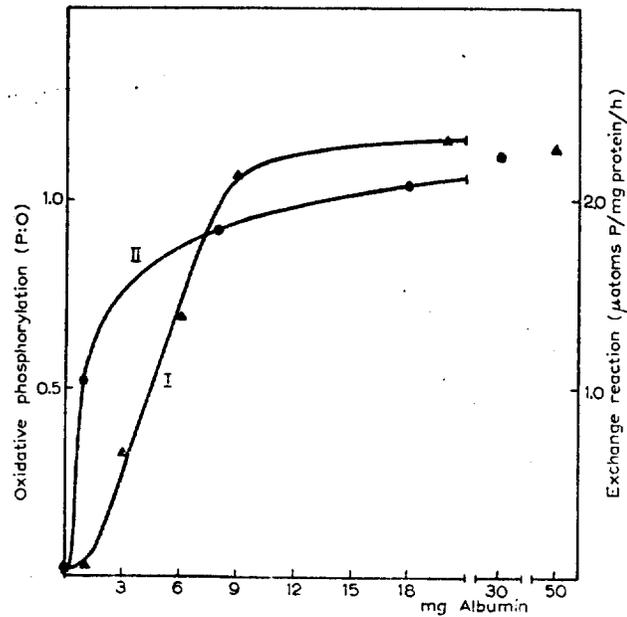


Fig. 1. Effect of various quantities of bovine plasma albumin on oxidative phosphorylation (Curve I) and ATP- P_i exchange (Curve II) in wax-moth mitochondria (substrate:succinate)

The stimulatory effect on the exchange reaction was not observed with any of the following amino acids tested separately: glycine, alanine, serine, cysteine, phenylalanine, tyrosine, tryptophan, histidine, methionine, valine, leucine, arginine, lysine, aspartic acid, glutamic acid*, threonine and proline (20 μ moles of each), nor with the peptides: glutathione (20 μ moles), carnosine (10 μ moles) and anserine (10 μ moles).

SACKTOR *et al.*⁵ have suggested that sulphhydryl groups of albumin are involved in the stimulatory effect on phosphorylation. This suggestion was based on the observation that albumin treated with pCMB had no stimulatory effect. In our experiments also, pCMB-treated albumin did not stimulate the exchange reaction. This was, however, due rather to an inhibition of the exchange reaction by pCMB bound with albumin than to a lack of stimulation since native and pCMB-treated albumins added together also did not increase the rate of the exchange reaction. Moreover, the iodination of albumin, which is known to oxidize sulphhydryl groups, did not appreciably diminish the stimulatory properties. All this suggests that free SH groups of albumin are not necessarily involved in the stimulation of ATP- P_i exchange in insect mitochondria.

ATPase activity

Wax-moth mitochondria contain a Mg-activated ATPase (on an average 3 μ moles ATP hydrolysed/mg mitochondrial protein/h) which is not further activated by 2,4-dinitrophenol. Serum albumin (4.5 mg/ml incubation medium) usually diminished this activity by a half. This effect of added albumin on ATPase activity was not

* 10^{-3} M KCN was present in the incubation medium, in this case to inhibit the oxidation of glutamate and to prevent the net phosphorylation which is coupled with that process.

always observed, however, which is in sharp contrast with the considerable and reproducible effect on the ATP-P₁ exchange reaction and on the net phosphorylation.

"Activation" of wax-moth mitochondria and isolation of an inhibitor of ATP-P₁ exchange

Further experiments showed that washing wax-moth mitochondria with an albumin-sucrose solution was almost as effective in stimulating ATP-P₁ exchange as was the addition of albumin to the reaction medium. In this procedure isolated mitochondria were suspended in 0.25 *M* sucrose containing 3% bovine or human serum albumin and centrifuged. They were washed subsequently with 0.25 *M* sucrose to remove the remaining albumin, centrifuged and resuspended in 0.25 *M* sucrose. Mitochondria which had been "activated" in this way catalysed on an average the exchange of 1.1 μ atoms P/mg mitochondrial protein/h, which is approximately half of the average exchange in the presence of 4.5 mg albumin in the incubation medium, but is many times greater than the exchange by "non-activated" mitochondria in the absence of albumin (*cf.* Table I). This state of "activation" was, however, transitory; the ability to catalyse the exchange reaction in the absence of albumin decreased on standing in sucrose solution at 0° and after storage for 1 h no "activation" could be observed.

Washing the mitochondria with a sucrose-albumin solution was also effective in promoting oxidative phosphorylation, although the P:O ratio was still rather low.

The sucrose-albumin solution that had been used to wash the mitochondria (henceforth termed "washing fluid") was heated for a few minutes at 100° in order to coagulate the albumin, and a portion of the suspension obtained was added to the incubation medium which contained "activated" wax-moth mitochondria. This resulted in a considerable decrease of ATP-P₁ exchange (Table II, Expt. 1). A similar inhibitory effect was also produced by the precipitate of coagulated albumin from the washing fluid but not by the clear supernatant obtained by centrifuging off the precipitated albumin. A control test showed that pure serum albumin if thermally coagulated had only a slight inhibitory effect.

TABLE II
INHIBITION OF ATP-P₁ EXCHANGE IN "ACTIVATED" WAX-MOTH MITOCHONDRIA

Expt.	Addition	Exchange (μ atoms P ₁ mg protein, h)	Inhibition %
1	None	0.80	
	"Washing fluid" (heated)	0.39	51
	Coagulated albumin from the washing fluid	0.22	73
	Albumin-free supernatant from the washing fluid	0.76	5
	Bovine serum albumin (thermally coagulated)	0.67	16
2	None	0.67	
	Ethanol extract	0.05	92
	Benzene extract	0.12	82
	Ethanol-extracted albumin from the washing fluid	0.65	3

In another experiment portions of the coagulated albumin from the washing fluid were extracted with organic solvents: ethanol, benzene and acetone. The extracts were placed in incubation tubes and the solvents were evaporated prior to the addition of incubation medium and mitochondria. It appeared (Table II, Expt. 2) that the extracts strongly inhibited the exchange reaction in "activated" mitochondria, whereas the albumin after extraction was without inhibitory effect.

On the basis of these results, the following routine procedure was adopted to isolate the inhibitory substance. Mitochondria from 50 larvae (about 70 mg mitochondrial protein) were suspended in 0.25 *M* sucrose and an equal volume of 0.25 *M* sucrose containing 6% serum albumin was added. The mitochondrial suspension was then allowed to stand for 30–60 min at 0° and centrifuged during 10 min at 12,500 × *g* in order to allow the mitochondria to settle. The supernatant fluid ("washing fluid") was decanted and heated for 5 min at 100°. It was then centrifuged and the precipitate of coagulated albumin was washed once with water and afterwards extracted with two portions of 1.5 ml ethanol. This ethanol extract contained the inhibitor of the ATP-P₁ exchange reaction; 0.3 ml of the extract was sufficient for complete inhibition of the exchange reaction with "activated" mitochondria in a usual test system.

Effect of the inhibitory substance on rat liver mitochondria

The substance obtained as described above appeared to inhibit the ATP-P₁ exchange reaction not only in "activated" wax-moth mitochondria but also in rat liver mitochondria. In addition, it uncoupled oxidative phosphorylation and stimulated the latent ATPase of liver mitochondria, as is shown in Table III. All these effects could be abolished by the addition of serum albumin.

TABLE III
EFFECT OF ETHANOL EXTRACT FROM THE WASHING FLUID ON PHOSPHORYLATION COUPLED TO THE OXIDATION OF GLUTAMATE, ATP-P₁ EXCHANGE AND ATPase ACTIVITY IN RAT-LIVER MITOCHONDRIA

Additions	Oxidative phosphorylation (P:O)	Exchange reaction (atoms P/mg protein/h)	ATPase activity (μmoles/mg protein/h)
None	2.5	2.3	0.2
Ethanol extract, 0.5 ml	0.1	0.1	2.0
Ethanol extract, 0.5 ml and serum albumin, 0.0 mg	2.8	1.7	0.0

Ethanol was evaporated prior to the addition of incubation medium and mitochondria.

Preliminary experiments were made to investigate a possible effect of this substance on ATP-ADP exchange. This reaction may proceed not only as a result of mechanisms related to oxidative phosphorylation¹⁸ but also as result of some other enzymic processes. In our experiments the activity of adenylyate kinase was partly inhibited when fluoride was included in the medium. It was shown in a separate experiment that the remaining activity of adenylyate kinase was responsible for not more than 10% of the exchange observed. Participation of other mechanisms that might be expected to convert labeled ADP to ATP (*cf.* WADKINS AND LEHNINGER¹⁹), however, could not be excluded in the present investigations. The results have therefore only a tentative character.

The ATP-ADP exchange reaction in rat liver mitochondria appeared to be several times more intense than the ATP- P_i exchange reaction and, in contrast to the latter, was not inhibited by the ethanol extract obtained as described in the preceding section. ATP-ADP exchange in wax-moth mitochondria was almost equally intense in the absence as in the presence of serum albumin.

Chemical characterization of the inhibitor of the ATP- P_i exchange reaction

The solubility in organic solvents suggested that the inhibitory substance was of a lipid character. Preliminary experiments showed that the inhibitor was not destroyed by heating in 1 *N* HCl or 1 *N* KOH for 3 h at 100°. This suggested that it was not fat, or phospholipid or other hydrolysable compound. A portion of the ethanol extract containing the inhibitor was made alkaline with 6 *N* KOH and, after dilution with water, extracted with ether (extract I). The water phase was acidified with HCl and again extracted with ether (extract II). Extract I, which could contain fats, phospholipids and unsaponifiable material, was without effect on the ATP- P_i exchange reaction in rat liver mitochondria, while extract II was strongly inhibitory. This extract might contain fatty acids.

A small drop of an ethanol solution of the inhibitor placed on a piece of filter paper gave positive colour tests for fatty acids in the copper acetate-potassium ferricyanide procedure and for unsaturated fatty acids with alkaline permanganate solution. On chromatography in 93% acetic acid, one spot was detected by copper acetate-potassium ferricyanide treatment that corresponded to palmitic or/and oleic acids. Occasionally another spot corresponding to stearic acid could be observed with more concentrated extracts of the inhibitor. With an alkaline permanganate solution three spots were distinctly visible. The most proximate to the starting line, with the same R_F value as the spot found with copper acetate-potassium ferricyanide, corresponded to oleic acid; the two remaining spots had R_F values of linoleic and linolenic acids. These acids were probably present in far smaller quantities than oleic acid and could not be detected by the copper acetate-potassium ferricyanide procedure for fatty acids, which is less sensitive than the permanganate reagent for unsaturated bonds. In order to ascertain whether palmitic acid was also present, the inhibitor was chromatographed in acetic acid containing hydrogen peroxide*. Under these conditions unsaturated fatty acids are destroyed. Indeed, if a sufficient quantity of the inhibitor was present a distinct spot of palmitic acid and a faint one of stearic acid could be observed.

It is known¹⁹ that preparations of serum albumin usually contain small quantities of bound fatty acids. We have demonstrated, however, that the original content of fatty acids in the amount of albumin used in washing the mitochondria is too small to be detected chromatographically by the procedure adopted here.

An approximate evaluation of the amount of oleic acid present in the inhibitor was done by comparing the intensity of spots on chromatograms with spots given by known quantities of the acid. It appeared that about 0.7 mg of oleic acid could be obtained from mitochondria from 50 larvae by the procedure described above.

To make sure that the inhibitory effect was due to fatty acids the inhibitor was chromatographed, various parts of chromatogram were eluted with ether and the

* According to Dr. H. K. Maxam, Chemical Institute, Austin, Manitoba, private information from Prof. W. N. S. Pilbrow.

TABLE IV
INHIBITION OF ATP-P₁ EXCHANGE IN RAT LIVER MITOCHONDRIA BY ETHER ELUATES FROM CHROMATOGRAMS OF THE INHIBITING SUBSTANCE (MEAN VALUES FROM 5 EXPERIMENTS)

Part of chromatogram eluted	Inhibition %
Origin	4
Spots of fatty acids	59
Solvent front	43

Ether was evaporated prior to the addition of reaction mixture and mitochondria.

eluates tested for inhibition of ATP-P₁ exchange in rat liver mitochondria. It appeared (Table IV) that the eluates from the area containing fatty acids as well as from an area near the solvent front line produced a distinct inhibition, whereas the eluates from other parts of the chromatogram were without effect. It was also shown in a separate experiment that chemically pure salts of fatty acids inhibited ATP-P₁ exchange in rat liver mitochondria: 100 µg of K oleate were sufficient to block the exchange almost completely and 100 µg of Na stearate diminished the rate of the exchange reaction by half.

DISCUSSION

Serum albumin is without effect on oxidative phosphorylation in fresh mammalian liver mitochondria but, a pronounced protective effect has been observed on phosphorylation in "aged" mitochondria^{20,21}. This effect has been attributed²² to the binding by albumin of an uncoupling substance, a haemoprotein called "mitochrome", which is liberated from mitochondria during ageing. The active component of mitochrome has been shown¹² to be extractable with organic solvents and has been recently identified²³ as a mixture of fatty acids.

The present investigation shows that the effect of serum albumin on oxidative phosphorylation and ATP-P₁ exchange in wax-moth mitochondria is also due to the binding by albumin of an uncoupling substance which, in contrast to liver mitochondria, is present even in freshly prepared insect particles. This uncoupling agent can be isolated by washing wax-moth mitochondria with a sucrose-albumin solution and subsequent extraction of the washing fluid with organic solvents. The extract has been shown to contain fatty acids among which palmitic, stearic, linoleic and linolenic have been identified. It is thus evident that the composition of the uncoupling agent from insect mitochondria is similar to the composition of the lipid fraction of mitochrome as investigated by HÜLSMANN²³.

It is probable that besides the five fatty acids mentioned other active components are also present in the uncoupling substance from wax-moth mitochondria. This is indicated by inhibition of ATP-P₁ exchange produced by eluates from paper chromatograms of the inhibitor.

An explanation that the effect of some proteins on oxidative phosphorylation in insect mitochondria may be due to the binding of an uncoupling substance has been proposed some years ago by REES³ and has been supported recently by SACKROB²⁴ although no clear-cut experimental evidence has been given so far. Recently, Arai²⁵

AND GONDA²⁴ have demonstrated a stimulatory effect of serum albumin on the ATP-P_i exchange reaction in tissue particles from mosquitoes and house flies. This effect is produced if albumin is present in either the reaction medium or in the washing solution. This is very consistent with our previous report⁶ and with the present results with mitochondria from wax-moth larvae.

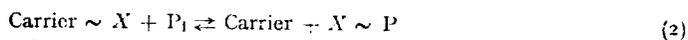
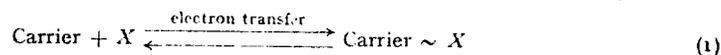
The fact that, among a variety of proteins investigated, serum albumin and β -lactoglobulin are most effective in promoting oxidative phosphorylation and stimulating the ATP-P_i exchange reaction can be understood on the basis that these two proteins have a very high binding property towards anions (*cf.* KLOTZ²⁵) and particularly towards fatty acids (*cf.* DEUEL²⁶).

There is no clear evidence why the isolation procedure that gives extensively phosphorylating mitochondria from mammalian liver or heart is usually unsuitable for isolation of phosphorylating particles from insects. One can suspect that some lipid constituents which give rise to free fatty acids are more labile in mitochondria from insects than from mammalian tissues or that enzymes are present in insect tissues which split mitochondrial lipids, with the liberation of free fatty acids. This question needs to be investigated further.

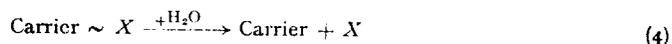
It is worth while mentioning that oxidative phosphorylation in particles from pigeon skeletal muscles²⁷, from rat brain²⁸ and from *Azotobacter vinelandii*²⁹ is also stimulated or protected by an addition of serum albumin. It may be supposed that the mechanism of this effect is similar to that acting in the case of insect mitochondria.

Uncoupling of oxidative phosphorylation and stimulation of latent ATPase by fatty acids have already been demonstrated by PRESSMAN AND LARDY^{30,31} with rat liver mitochondria. The present investigation shows, in addition, that fatty acids are inhibitory in ATP-P_i exchange, but probably not in ATP-ADP exchange reactions. The mechanism of these effects is not clear. It can be supposed that fatty acids may damage mitochondrial structure as surface-active agents³¹, thus uncoupling oxidative phosphorylation and stimulating latent ATPase, or that they have a more specific inhibitory effect on some enzymic processes involved in the mechanism of oxidative phosphorylation.

The following equations have been formulated¹⁸ for ATP synthesis coupled with electron transport:



Reactions (2) and (3) are involved in ATP-P_i exchange, reaction (3) in ATP-ADP exchange. Reactions (3) and (2) followed by an irreversible hydrolysis of Carrier ~ X:



are the basis of mitochondrial ATPase activity. The effects of fatty acids on oxidative phosphorylation, mitochondrial ATPase and ATP-P_i exchange may be explained by the assumption²³ that fatty acids, like other uncoupling agents, promote the irreversible reaction (4). Assuming that the rate of reaction (3) is far greater than that of reaction (2), no considerable inhibitory effect on ATP-ADP exchange would be expected — which is indeed the case.

It was observed in the present investigation that serum albumin increased the rate of substrate oxidation by wax-moth mitochondria in the presence of phosphate acceptor. Similar results have been obtained^{1,2,5,32} with mitochondria from other insects. This effect can also be accounted for on the basis of removal of fatty acids by added albumin. As assumed by HÜLSMANN²³, fatty acids not only promote splitting of the compound Carrier ~ X but also bind with free Carrier, thus lowering the "steady-state" concentration of the Carrier which is necessary for electron transfer (reaction (1)).

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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 ATPase³; (c) inhibition of the ATP-P_i exchange reaction^{4,5}; (d) uncoupling of oxidative phosphorylation^{3,4}; 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-DOR⁷.

The present paper describes a study on the swelling effect exerted by fatty acids of the saturated series over the range of C₃ to C₂₂ as well as by unsaturated acids and one hydroxy-monounsaturated acid.

Swelling of rat-liver mitochondria was measured photometrically at 520 m μ 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

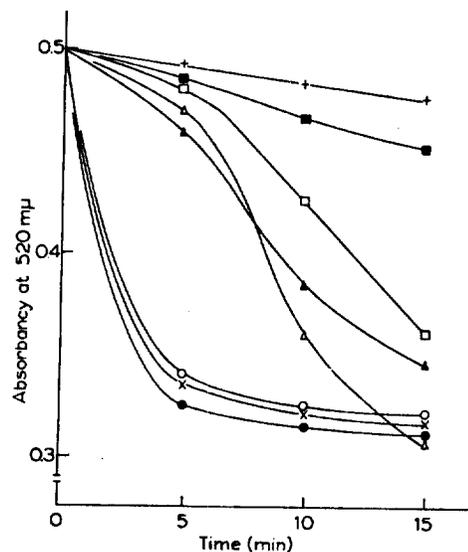


Fig. 1. Swelling of liver mitochondria induced by various fatty acids. Incubation mixture: 0.125 M KCl, 0.02 M Tris-HCl (pH 7.5), $1.33 \cdot 10^{-5}$ M sodium salts of fatty acids as indicated below, and rat-liver mitochondria containing about 5 mg protein; total volume, 7.5 ml; temperature, 20°. +—+, spontaneous swelling; x—x, oleic acid; □—□, caproic acid (C₆); Δ—Δ, caprylic acid (C₈); O—O, lauric acid (C₁₂); ●—●, myristic acid (C₁₄); ▲—▲, palmitic acid (C₁₆); ■—■, arachidic acid (C₂₀).

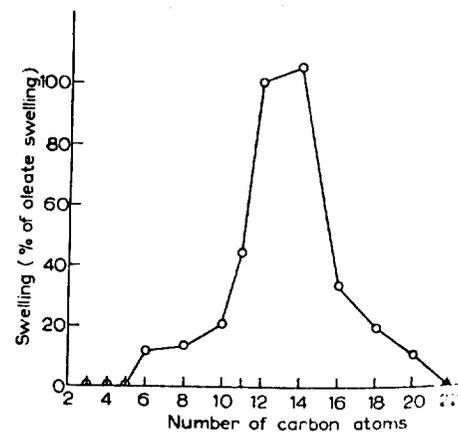


Fig. 2. Effect of saturated fatty acids on swelling of mitochondria from rat liver. Experimental conditions as in Fig. 1; incubation time, 5 min. Extent of swelling is expressed as per cent of the swelling produced by the same concentration of oleate during the same incubation time.

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	<i>cis</i> 18:1 ⁹	100
Elaidic acid	<i>trans</i> 18:1 ⁹	17
Linoleic acid	<i>cis cis</i> 18:2 ^{9,12}	98
Stearolic acid	18:1 ⁹	99
Ricinoleic acid	<i>cis</i> 18:1 ⁹ hydroxy ¹²	111
Behenic acid	22:0	0
Erucic acid	<i>cis</i> 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-DOR⁷, 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 LARDY³ 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 ones. A similar dependence upon the number of carbon atoms has also been observed with respect to the inhibition of the ATP-P₁ exchange reaction⁸, 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 BORST *et al.*⁹. All these facts support the view¹¹

that swelling of mitochondria is connected with the mechanism of coupling the electron transfer to the synthesis of ATP.

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SUGAR AND CORONARY HEART DISEASE

Cigarette smokers use more sugared hot beverages than nonsmokers, an observation which confounds a superficial association between sugar and coronary heart disease.

Among the nutritional diversions of the last decade has been the hypothesis, promulgated mainly by J. Yudkin (Yudkin and J. Morland, *Am. J. Clin. Nutrition* 20, 503 (1967)), that dietary sugar is a major factor in the contemporary epidemic of coronary heart disease (CHD). Historically, and even today, nutrition has been susceptible to anecdotal or tenuously founded claims in regard to special (either beneficial or deleterious) properties of particular foods or nutrients—claims easily raised and publicized but rather more difficult to test in a rigorous manner. When coupled with CHD, a disease with a strong nutritional component but with the additional complex involvement of other host and environmental factors, one is faced with a very formidable combination indeed, for there is the continuing temptation to look for simplistic, single-factor solutions which will at once explain away the many apparent mysteries of atherosclerosis and its clinical complications.

The basis for the sugar-CHD link derived from the association between the parallel secular trends both in sugar con-

sumption and in mortality from CHD over the past 50 years and was supported by comparisons of sugar use among survivors of myocardial infarction and controls (Yudkin and J. Roddy, *Lancet* 2, 6 (1964)). Recently, two more carefully designed population studies have cast some doubt on the concept of a direct link and have suggested that cigarette smoking may be the operative intermediary factor. It should be pointed out that there still are inconsistencies in the epidemiologic evidence linking smoking to CHD (C. C. Seltzer, *J. Am. Med. Assn.* 203, 193 (1968); *Arch. Environ. Health* 29, 418 (1970)).

One of these two newer reports (P. C. Elwood *et al.*, *Lancet* 1, 1014 (1970)) based on two community surveys. Among women, 2,834 of 3,149 subjects 20 through 64 years of age in a defined population group were questioned about age, body weight, sugar consumption, the symptoms of angina pectoris, and cigarette smoking habits. Smokers used more sugar per day than nonsmokers and women with angina pectoris used about 10 g. per day more sugar than those without. The latter difference was not statistically significant.

nor was sugar usage consistently related to the amount of cigarette smoking. As a check on the reliability of the estimates of sugar consumption, 100 women were re-surveyed two years later and the simple correlation coefficient between the two estimates was 0.8 ($p < 0.001$).

The male community survey included 344 men (79 per cent of an age and occupation stratified sample who had been examined in 1958). Electrocardiograms as well as the same questions used in the female sample were collected. Neither on the basis of angina pectoris nor on the basis of the electrocardiographic patterns was there any demonstrable relationship between consumption of sugar and CHD. Here too, sugar consumption per day was greater among pack-a-day smokers (103 g.) than nonsmokers (83 g.). Thus both of these studies suggested a relationship between the use of sugar and of smoking habits, a confounding factor in relating sugar to CHD.

A companion article by A. E. Bennett, R. Doll, and R. W. Howell (*Lancet* 1, 1011 (1970)) lends further support to this observation. This report is actually of three separate surveys. Among male hospitalized patients aged 35 through 64, mean daily sugar use in hot beverages ranged from 34.6 g. among nonsmokers to 53.4 among smokers of 15 or more cigarettes per day. Data on 2,483 males 40 through 54 years of age (representing a 90

per cent response of a random sample of industrial workers) related smoking habits to a questionnaire-derived estimate of sugar consumption. Total daily sugar use was higher among cigarette smokers than nonsmokers, the difference being accounted for by sugar consumption in hot drinks. Lastly, a random sample of 453 males 26 through 65 years of age residing in a defined area also yielded data showing that heavy cigarette smokers used more hot beverages (8.1 cups per day compared with 5.2 in light smokers and 4.8 in nonsmokers) as well as more sugar per cup (9.1 g. among heavy smokers, 7.8 g. among light smokers). Considering all of these studies together, there was noted a significant association ($p < 0.05$) between cigarette smoking and both hot beverage use and sugar consumption.

Various speculations about this association were raised by Bennett *et al.* Are both smoking and tea or coffee drinking related to some underlying psychological need or are they merely socializing habits shared by chance? Could oral-pharyngeal dryness in smokers lead to a greater use of beverages? Does the greater use of sugar per cup of hot beverage among smokers suggest an altered taste threshold for sweetness? Whatever the mechanisms involved, it now appears that the apparent association between CHD and sugar is neither as straightforward nor as clear as originally hypothesized.